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Two Critical Periods of Sonic Hedgehog Signaling Required for the Specification of Motor Neuron Identity

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Summary

Antibodies that block Sonic Hedgehog (SHH) signaling have been used to show that SHH activity is required for the induction of floor plate differentiation by the notochord and independently for the induction of motor neurons by both the notochord and midline neural cells. Motor neuron generation depends on two critical periods of SHH signaling: an early period during which naive neural plate cells are converted into ventralized progenitors and a late period that extends well into S phase of the final progenitor cell division, during which SHH drives the differentiation of ventralized progenitors into motor neurons. The ambient SHH concentration during the late period determines whether ventralized progenitors differentiate into motor neurons or interneurons, thus defining the pattern of neuronal cell types generated in the neural tube.

Introduction

The patterning of cell types in vertebrate embryos depends on the function of organizing centers, specialized cell groups that direct the fate of nearby cells through the secretion of inductive factors (Gurdon, 1987). The identity and pattern of cell types generated in the ventral neural tube is controlled initially by an axial mesodermal organizing center, the notochord (Jessell and Dodd, 1992). The notochord secretes a locally acting factor that induces the differentiation of floor plate cells at the ventral midline of the neural tube and a diffusible factor that can initiate motor neuron differentiation (reviewed by Placzek, 1995).

The secreted glycoprotein Sonic Hedgehog (SHH) is expressed by the notochord and later by the floor plate and is a strong candidate as a local inducer of floor plate differentiation (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995; Hynes et al., 1995b; Ericson et al., 1995; Marti et al., 1995a; Tanabe et al., 1995). Exposure of neural plate cells to the biologically active amino-terminal fragment of SHH (SHH-N) (Lee et al., 1994; Porter et al., 1995) is sufficient to induce expression of floor plate markers, notably the transcription factor HNF3β (for hepatocyte nuclear factor 3β; Roelink et al., 1995; Hynes et al., 1995b). The induction of HNF3β in neural plate cells is an early response to notochord-derived signals (Ruiz i Altaba et al., 1995a), and ectopic expression of HNFβ induces floor plate differentiation (Ruiz i Altaba et al., 1993, 1995b; Sasaki and Hogan, 1994; Hynes et al., 1995a). These findings suggest that in vivo, the high level of SHH associated with the surface of notochord cells (Marti et al., 1995b; Roelink et al., 1995) exposes midline neural plate cells to a concentration of SHH-N above the threshold for induction of HNF3β, leading subsequently to the expression of other floor plate genes. Nevertheless, it remains to be shown that SHH is required for the notochord-mediated induction of floor plate differentiation.

The role of SHH in the specification of motor neuron identity is less clear. In part, this uncertainty reflects the fact that the generation of motor neurons is a protracted process. Neural plate cells are exposed to signals from the notochord soon after neural plate formation (Liem et al., 1995), but postmitotic motor neurons are first generated ~18 hr later (Ericson et al., 1992; Pfaff et al., 1996), after neural plate cells have undergone one or more rounds of cell division (Langman et al., 1966). Thus, even though SHH is able to induce motor neurons in vitro at concentrations below the threshold for floor plate differentiation (Marti et al., 1995a; Roelink et al., 1995; Tanabe et al., 1995), it remains possible that SHH induces an intermediary factor in neural plate cells and that this factor rather than SHH itself is responsible for motor neuron differentiation. SHH secreted from the notochord might therefore control cell pattern in the ventral neural tube entirely through local signaling, with long-range cell patterning mediated by a secondary diffusible factor. Indeed, many of the long-range patterning activities of Hedgehog in Drosophila depend on the induction of diffusible intermediary factors, notably the transforming growth factor β (TGFβ)-like protein Decapentaplegic (DPP) (Zecca et al., 1995; Nellen et al., 1996; Lecuit et al., 1996). Antibodies directed against SHH have been shown to inhibit the induction of motor neuron differentiation by the notochord in neural plate explants (Marti et al., 1995a). However, the notochord normally induces floor plate cells under these conditions, and thus it remains unclear whether the requirement for SHH signaling is in floor plate differentiation, motor neuron differentiation, or both.

In this study, we have addressed whether SHH is required for the generation of floor plate cells and motor neurons by blocking SHH signaling in vitro with anti-SHH antibodies. We show that SHH function is required for the short-range induction of floor plate cells by the notochord and independently for the induction of motor neurons both by the notochord and midline neural cells.

These results raise the additional question of when and over what range SHH signaling is required for the generation of motor neurons. We provide evidence that motor neuron generation depends on two critical periods of SHH signaling, with long range actions at different concentration thresholds. During the early period, SHH derived from the notochord converts naive neural plate

cells into ventralized progenitors. During the late period, which extends well into S phase of the final progenitor cell division, SHH derived from the floor plate acts at a distance to direct the differentiation of ventralized progenitors into postmitotic motor neurons. These results provide strong evidence against a cascade model in which a SHH-inducible intermediary factor is sufficient for motor neuron differentiation. The ambient SHH concentration during the late period determines whether ventralized progenitors differentiate into motor neurons or interneurons, thus defining the pattern of differentiation of specific neuronal cell types in the ventral neural tube.

Results

SHH Is Required for the Induction of Floor Plate and Motor Neuron Differentiation

To determine whether SHH is required for the induction of ventral cell types in the caudal neural tube, we generated polyclonal (H4) and monoclonal (MAb 5E1) antibodies against the biologically active amino-terminal fragment of SHH (SHH-N). The H4 and 5E1 antibodies recognize the ~20 kDa SHH-N protein by Western blotting and enzyme-linked immunosorbent assay (data not shown). Both antibodies also react with Indian Hedgehog (IHH) (Vortkamp et al., 1996), but since neither IHH nor Desert Hedgehog (DHH) is expressed by notochord or neural tube cells (Bitgood et al., 1996; data not shown), in the present studies the H4 and 5E1 antibodies recognize SHH selectively.

Affinity-purified H4 immunoglobulin G (IgG) was tested first for its ability to block the SHH-N-mediated induction of floor plate and motor neuron differentiation in intermediate neural plate ([I]) explants (Yamada et al., 1993). Explants exposed to recombinant SHH-N (4 nM) for 48 hr gave rise to HNF3β$^+$ floor plate cells and Isl1/Isl2$^+$ motor neurons (Figures 1A and 1E). Addition of H4 IgG blocked the SHH-N-mediated induction of floor plate cells and motor neurons (Figures 1B and 1F; data not shown). Three results demonstrate the specificity of action of the H4 antibody. First, increasing the concentration of SHH-N to 13 nM in the presence of H4 IgG restored motor neuron differentiation (205 ± 27 Isl1/Isl2$^+$ cells, n = 5). Second, the blocking activity of H4 was mimicked by MAb 5E1 IgG (0 ± 0 Isl1/Isl2$^+$ cells, n = 5), but not by rabbit-anti SHH (H2) IgG (data not shown), a reagent that reacts only with denatured SHH protein (Roelink et al., 1995). Third, H4 IgG had no effect on the bone morphogenetic protein 4 (BMP4)-mediated induction of neural crest cell differentiation in [I] explants (data not shown; see Liem et al., 1995).

To examine whether induction of floor plate differentiation by the notochord is mediated by SHH, we grew conjugates of notochord and [I] explants in the presence or absence of H4 IgG. Floor plate differentiation, detected by HNF3β expression, was blocked in the presence of H4 IgG (Figures 1C and 1D). These results indicate that SHH is required for the notochord-mediated induction of floor plate differentiation.

To determine whether motor neuron generation requires SHH signaling, previously described midline sources of motor neuron–inducing factors (Placzek et al., 1991; Yamada et al., 1993) were tested for their sensitivity to anti-SHH antibodies. The notochord-mediated induction of motor neurons was blocked by H4 IgG (Figures 1G and 1H), consistent with previous results (Marti et al., 1995a). Motor neuron induction by the notochord under transfilter conditions that reveal the activity of a diffusible factor (Tanabe et al., 1995) was also blocked by H4 IgG (Figures 1I and 1J). In addition, motor neuron induction by the floor plate or by stage 10 ventral midline tissue was blocked by H4 IgG (Figures 1K and 1L; see also Figure 5A). In contrast, H4 IgG did not affect the ability of the floor plate to induce commissural axon extension from rat E11 dorsal spinal cord explants in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990; data not shown), an activity mediated by netrins (Serafini et al., 1994). These results provide evidence that SHH signaling is required for the induction of motor neuron differentiation by both midline mesodermal and neural cells.

Early Action of Notochord-Derived SHH on Neural Plate Cells

To begin to determine when SHH signaling is required for motor neuron generation, we analyzed the time at which neural plate cells are first exposed to a SHH-mediated signal. Previous studies have indicated that notochord-derived signals repress expression of the homeobox genes *pax3* and *msx1* from medial regions of the neural plate (Liem et al., 1995). PAX7 is also initially expressed in some cells at the midline of the newly formed neural plate (data not shown), but its expression is lost rapidly from medial neural plate cells (Figure 2B) and subsequently is restricted to the dorsal neural tube (Figure 2D). To determine whether signals from the notochord are responsible for the absence of PAX7 expression, in medial neural plate cells, stage 10 [I] explants (Figure 2E) were grown alone or with the notochord for 22 hr. Over 95% of cells in explants grown alone expressed PAX7 (Figure 2F), whereas expression was eliminated from neural plate cells grown in contact with the notochord (Figure 2G). The notochord-mediated repression of PAX7 expression was mimicked by SHH (Figure 2H) and blocked by H4 IgG (Figure 2I). Thus, SHH is necessary and sufficient for the notochord-mediated repression of PAX7 expression in neural plate cells in vitro. The absence of PAX7 expression by medial neural plate cells in vivo therefore shows that these cells have been exposed to notochord-derived SHH at the time of neural plate formation.

We next examined whether persistent SHH signaling is required to maintain the repression of PAX7 expression. To test this, stage 10 ventral neural plate explants were isolated at a stage at which expression of PAX7 had already been extinguished (Figure 2J) and were grown in vitro for 22 hr. Reexpression of PAX7 was detected in >95% of cells (Figures 2K and 2L), suggesting that cells in the medial neural plate require continued SHH signaling from the notochord to maintain the repression of PAX7 expression. In contrast, cells in explants derived from stage 12 ventral neural tube devoid of floor plate (Figure 2E) did not reexpress PAX7

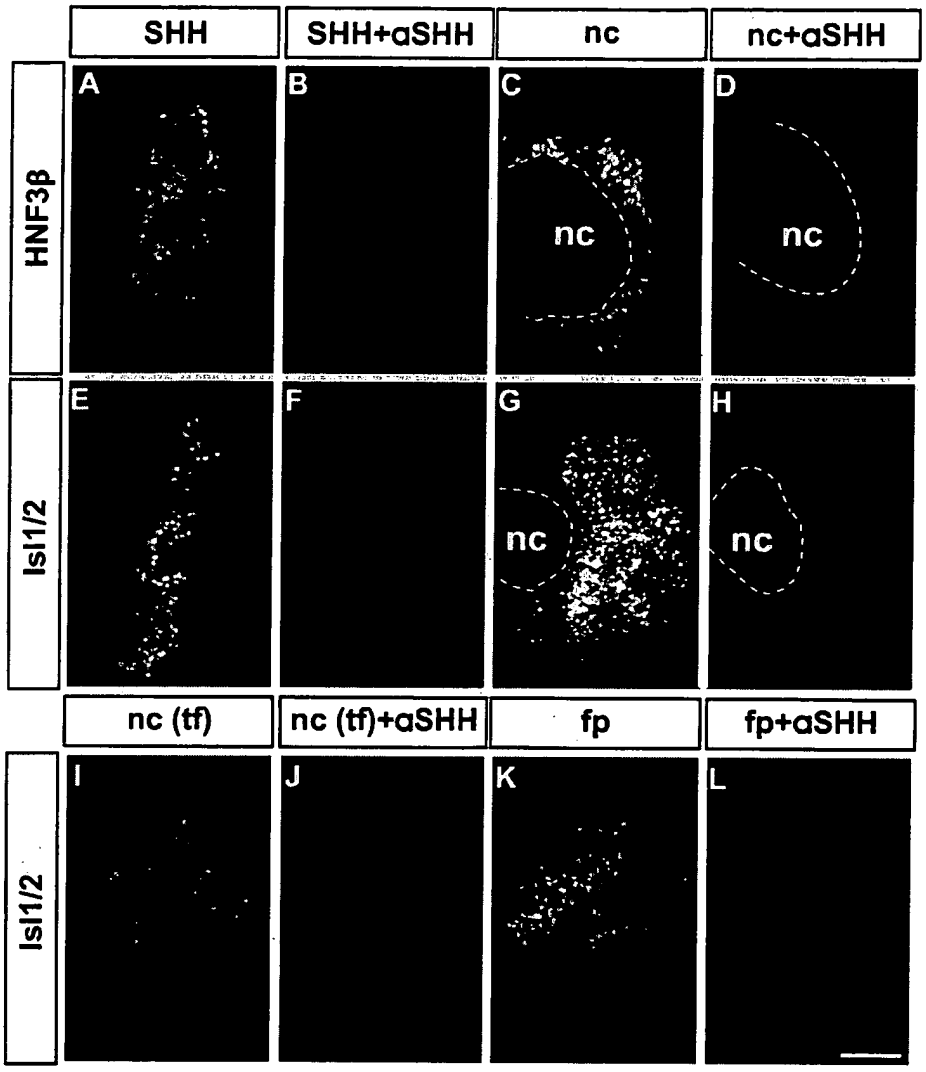

Figure 1. Blockade of Floor Plate and Motor Neuron Differentiation by Anti-SHH Antibodies

Stage 10 [I] explants were grown for 48 hr.

(A and B) Induction of HNF3β$^+$ cells by SHH-N (4 nM) (149 ± 20 cells, n = 6; [A]) is blocked by H4 IgG (0 cells, n = 6; [B]).

(C and D) Notochord-mediated induction of HNF3β$^+$ cells (168 ± 19, n = 8; [C]) is blocked by H4 IgG (0 cells, n = 15; [D]).

(E and F) Induction of Isl1/Isl2$^+$ cells by SHH-N (4 nM) (383 ± 20 cells, n = 8; [E]) is blocked by H4 IgG (0 cells, n = 9; [F]).

(G and H) Notochord-mediated induction of Isl1/Isl2 neurons (282 ± 41, n = 10; [G]) is blocked by H4 IgG (8 ± 4 cells, n = 10; [H]).

(I and J) Notochord transfilter induction of Isl1/Isl2 neurons (27 ± 10, n = 6; [I]) is blocked by H4 IgG (0 cells, n = 10 explants; [J]).

(K and L) Floor plate induction of Isl1/Isl2 neurons (144 ± 13, n = 6; [K]) is blocked by H4 IgG (1 ± 1 cells, n = 10; [L]).

Values indicate mean ± SEM. Scale bar, 120 μm.

when grown alone (Figures 2M and 2N), or in the presence of H4 IgG (Figure 2O). Thus, by stage 12 the extinction of PAX7 expression by ventral neural tube cells is independent of SHH signaling.

This analysis of PAX7 expression suggests that the development of neural plate cells into motor neurons is divisible into two distinct periods, an early (stage 10 to stage 12) period during which naive (PAX7^{on}) neural plate cells are converted to stable ventralized (PAX7^{on}) progenitors and a late (post–stage 12) period during which ventralized progenitor cells give rise to postmitotic motor neurons. The appreciation of this subdivision has permitted us to examine more precisely the temporal requirement for SHH signaling in motor neuron generation.

Early Exposure of Neural Plate Cells to SHH Is Required for Floor Plate and Motor Neuron Differentiation

To determine whether SHH signaling during the early period is required for the eventual differentiation of ventral cell types, stage 10 [I] explants were exposed to SHH-N for different periods of time, and floor plate and motor neuron generation was assessed. Exposure of

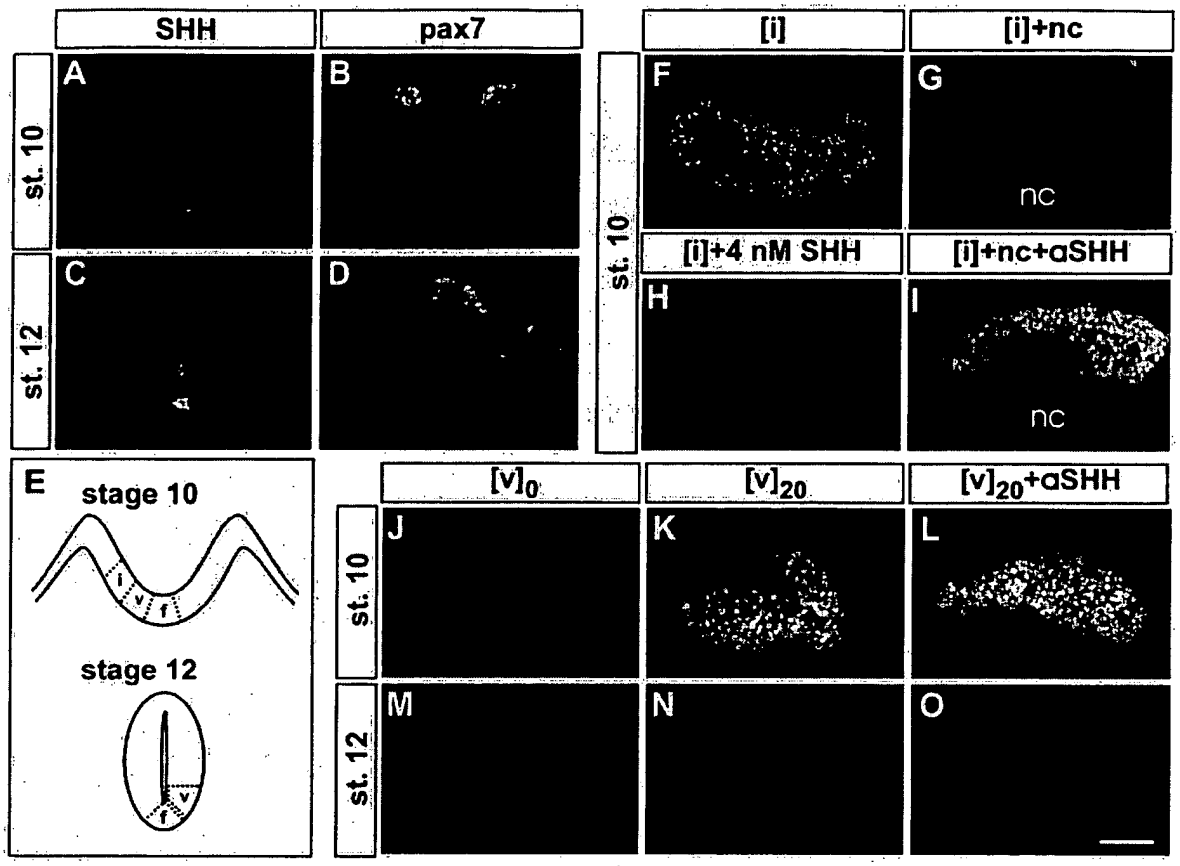

Figure 2. Notochord-Derived SHH Represses PAX7 Expression from Ventral Neural Cells

(A) SHH is expressed in the notochord but not by midline neural plate cells at stage 10.
(B) PAX7 expression is extinguished in the medial neural plate at stage 10.
(C) SHH is expressed by floor plate cells at stage 12.
(D) PAX7 expression is restricted to the dorsal neural tube at stage 12.
(E) Regions isolated for use in explant assays.
(F) PAX7 is expressed by >95% of cells in [i] explants grown alone for 22 hr.
(G) SHH-N (4 nM) represses PAX7 expression.
(H) Notochord represses PAX7 expression.
(I) H4 IgG blocks the notochord repression of PAX7 expression. PAX7 is detected in >90% of cells in neural plate explants after 22 hr.
(J) Cells in stage 10 ventral neural plate explants do not express PAX7 at the time of isolation.
(K) Over 90% of cells in stage 10 [v] explants reexpress PAX7 after 22 hr.
(L) H4 IgG does not influence reexpression of PAX7 in stage 10 [v] explants.
(M) Cells in stage 12 [v] explants do not express PAX7 at the time of isolation.
(N) Cells in stage 12 [v] explants do not reexpress PAX7 after 22 hr in vitro.
(O) Addition of H4 IgG does not result in reexpression of PAX7 in stage 12 [v] explants after 22 hr.
Results derived from five to ten explants. Scale bar in (A)–(D), 150 μm; in (F)–(O), 100 μm.

explants to 4 nM SHH-N resulted in the detection of many floor plate cells and motor neurons by 25 hr (Figures 3A and 3E). In contrast, explants grown in the absence of SHH-N for the first 12 hr followed by addition of 4 nM SHH-N for an additional 25 hr did not contain floor plate cells or motor neurons at 37 hr (Figures 3B and 3F). Thus, neural plate cells rapidly lose the competence to generate floor plate cells and motor neurons unless exposed to SHH during this critical early period. Repression of PAX7 expression was detected in all cells in explants exposed to 4 nM SHH for 12 hr, whereas in the absence of SHH, maintained PAX7 expression was detected in over 95% of cells (data not shown).

These observations raised the issue of whether the concentration of SHH necessary to maintain the competence of neural plate cells for floor plate and motor neuron generation during this early period is the same as that required for the eventual differentiation of these ventral cell types. To test this, neural plate explants were exposed to 0.4 nM SHH-N for 37 hr. At this concentration no floor plate cells or motor neurons were generated (Figures 3C and 3G). In contrast, exposure of neural plate explants to 0.4 nM SHH-N for 12 hr followed by addition of 4 nM SHH-N for an additional 25 hr resulted in the generation of both floor plate cells and motor neurons at 37 hr (Figures 3D and 3H). Exposure of explants to 0.4 nM SHH-N for 12 hr repressed PAX7 expression in ~50% of cells (data not shown).

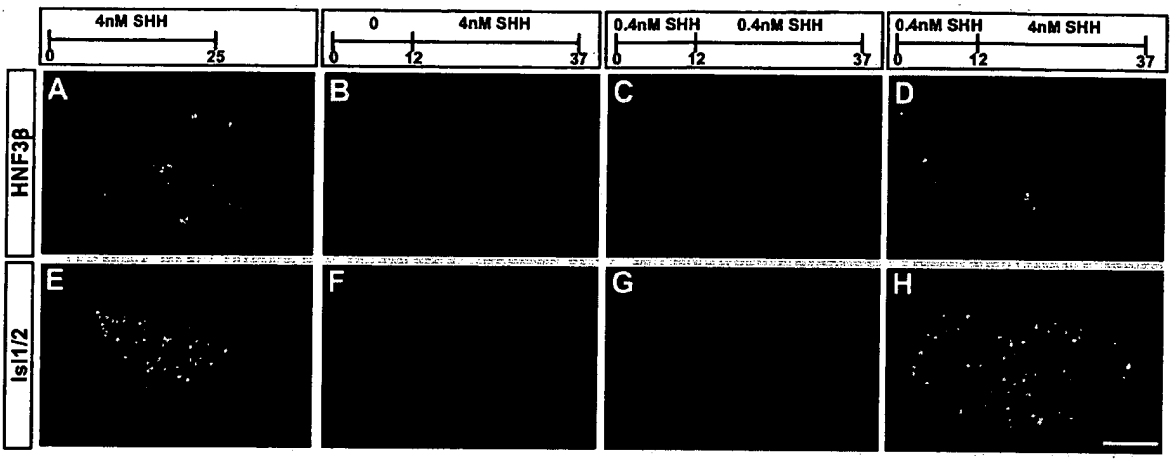

Figure 3. Early Requirement for SHH in Floor Plate and Motor Neuron Generation

(A and E) Floor plate cells (A) and motor neurons (E) are generated in stage 10 [i] explants grown in 4 nM SHH-N for 25 hr (150 ± 17 HNF3β$^+$ cells, n = 5; 118 ± 14 Isl1/Isl2$^+$ cells, n = 6) or 37 hr (168 ± 15 HNF3β$^+$, n = 7; 375 ± 40 Isl1/Isl2$^+$ cells, n = 4).

(B and F) Neither floor plate cells (B) (0 HNF3β$^+$ cells, n = 6) nor motor neurons (F) (1 ± 0 cells, n = 7) are generated when [i] explants are grown for 12 hr in the absence of SHH-N followed by 25 hr in 4 nM SHH-N. Similar results were obtained when the period in 4 nM SHH-N was extended to 33 hr (2 ± 1 Isl1/Isl2$^+$ cells, n = 6) or with 12 nM SHH-N (1 ± 1 Isl1/Isl2$^+$ cells; n = 5).

(C and G) Neither floor plate cells (C) (0 HNF3β$^+$ cells, n = 6) nor motor neurons (G) (0 cells, n = 6) are generated when [i] explants are grown for 37 hr in 0.4 nM SHH-N.

(D and H) Floor plate cells (D) (83 ± 10 HNF3β$^+$ cells, n = 7) and motor neurons (H) (211 ± 32 Isl1/Isl2$^+$, n = 7) are generated when [i] explants are grown for 12 hr in 0.4 nM SHH-N followed by 25 hr in 4 nM SHH-N.

These observations provide evidence that naive neural plate cells require exposure to SHH, acting at a low concentration threshold, during a critical period soon after neural plate formation to maintain the capacity to generate floor plate cells and motor neurons. They also show that the early repression of PAX7 expression by neural plate cells predicts their competence for later floor plate and motor neuron generation.

Ventralized Progenitors Require Additional SHH Signaling for Motor Neuron Generation

We next examined whether neural cells that have been converted to a ventralized state in vivo are able to differentiate into floor plate cells or motor neurons in the absence of further SHH signaling. Ventral midline neural plate tissue (f region, see Figure 2E) isolated from stage 10 embryos generated numerous HNF3β$^+$ and SHH$^+$ cells when grown alone or in the presence of H4 IgG for 24 hr (Figures 4A–4D). This result indicates that midline cells have been exposed to SHH for a period of time sufficient to specify floor plate identity shortly after neural plate formation.

To determine whether the early exposure of adjacent ventral neural cells to SHH is also sufficient to specify motor neuron identity, ventral neural tube explants devoid of midline tissue (v region, Figure 2E) were isolated at stage 12 and grown in vitro for 24 hr. Cells in such explants did not express HNF3β nor *Shh* mRNA (Figures 4G and 4H) and did not contain detectable SHH immunoreactivity (Figures 2A and 2C; data not shown). Nevertheless, these explants generated ~50 motor neurons (Figure 4E), raising the possibility that some ventralized progenitors are able to differentiate into motor neurons independent of further SHH signaling. This is not the case, however, since motor neuron generation in such explants was blocked by addition of H4 IgG (Figure 4F). Thus, the differentiation of ventralized progenitor cells into motor neurons requires additional SHH signaling. In addition, since SHH synthesis is restricted to notochord and floor plate cells, the blockade of motor neuron generation by anti-SHH antibodies provides strong evidence that the SHH protein required locally for the conversion of ventralized progenitor cells into motor neurons is obtained by diffusion from midline cells.

Prolonged Requirement for Floor Plate–Derived SHH in the Conversion of Ventralized Progenitors Into Motor Neurons

To test whether floor plate cells can serve as a source of SHH required for the conversion of adjacent ventralized progenitors into motor neurons, we isolated explants comprising the prospective or definitive floor plate together with flanking ventral neural tissue from stage 10–17 embryos and analyzed floor plate and motor neuron differentiation after 24–48 hr in vitro. HNF3β$^+$ floor plate cells and SHH expression were detected in these explants, and many motor neurons were generated (Figure 5). Addition of H4 IgG completely blocked motor neuron generation in explants derived from stage 10–12 embryos (Figures 5A and 5B). In contrast, floor plate differentiation, assessed by HNF3β and SHH expression, was not inhibited (Figures 4A–4D; data not shown). Thus, the blockade of motor neuron differentiation in the presence of anti-SHH antibodies does not result from the inhibition of floor plate differentiation or of SHH expression.

Motor neuron generation was markedly reduced but not completely abolished by addition of H4 IgG to explants derived from stage 13 and 14 embryos (Figures 5A and 5B). This observation raised the question of why some ventral progenitor cells in explants isolated at stage 13 (about 2 hr before the appearance of Isl1/Isl2$^+$

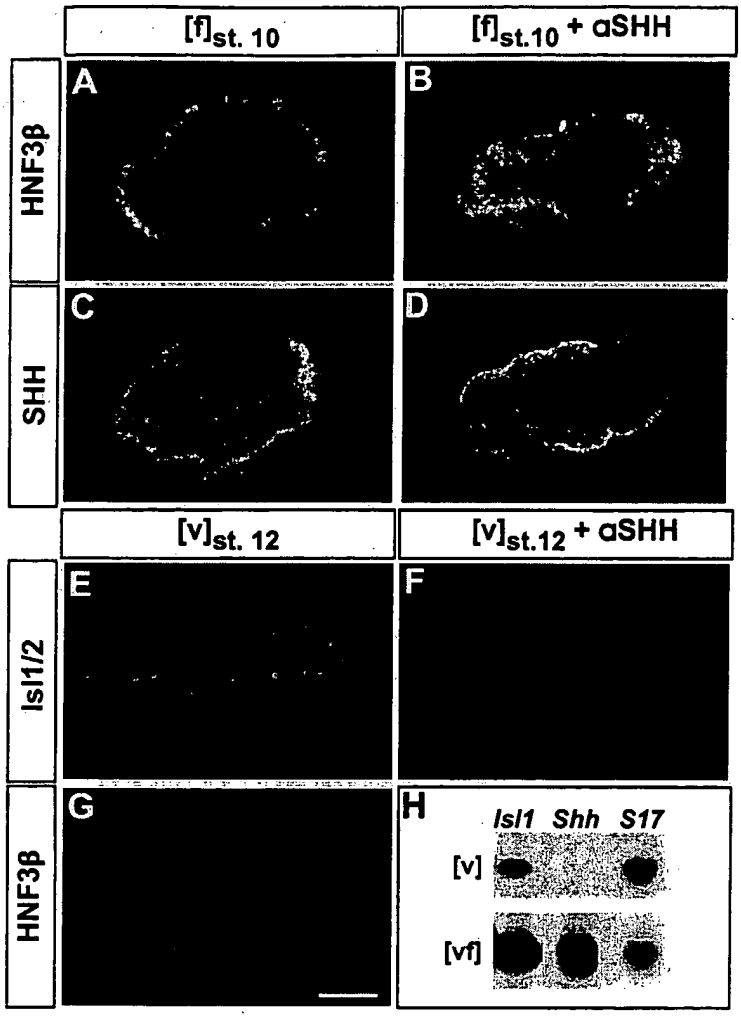

Figure 4. Ventralized Progenitor Cells Require Additional SHH Signaling for Motor Neuron Generation

(A and B) HNF3β$^+$ cells are generated in stage 10 [f] explants grown for 36 hr in the absence (A) (211 ± 19 cells; n = 6) or presence (B) (171 ± 16 cells, n = 6) of H4 IgG.

(C and D) SHH-immunoreactive cells are detected in [f] explants grown for 24 hr in the absence (C) or presence (D) of H4 IgG.

(E) Stage 12 [v] explants isolated in the absence of floor plate cells generate motor neurons (54 ± 10 cells, n = 7 explants) after 36 hr.

(F) Stage 12 [v] explants grown in the presence of anti-SHH H4 IgG do not generate motor neurons (0 cells, n = 7 explants).

(G) Stage 12 [v] explants do not contain HNF3β$^+$ cells.

(H) RT–PCR analysis of *Isl1*, *Shh*, and *S17* expression in stage 12 [v] or [vf] explants grown for 24 hr in vitro. This RT–PCR assay is sensitive at *Shh* transcript levels 200 fold below those present in floor plate cells. Similar results were observed in two assays.

Scale bar in (A)–(D), 70 μm; in (E), (G), and (H), 60 μm.

motor neurons; Figure 5B; data not shown) were able to generate motor neurons in a SHH-independent manner. We reasoned that the SHH-independent progenitors might be most advanced in their developmental program and destined to give rise to the first motor neurons detected in vivo. A prediction of this reasoning is that the first motor neurons to be generated in vitro in stage 13 floor plate/ventral explants should be insensitive to blockade by anti-SHH antibodies. Consistent with this premise, the generation of motor neurons over the first 10 hr period was not inhibited by the addition of H4 IgG, whereas motor neuron generation over the subsequent 26-hr period was completely blocked (Figure 6A). The lack of an early inhibitory effect of H4 IgG does not result from a delay in the blockade of SHH signaling, since motor neuron generation over the first 10 hr in stage 12 floor plate/ventral explants was inhibited (Figure 5B and data not shown). These results suggest that the inability of H4 IgG to block motor neuron generation completely at stages 13 and 14 can be explained by the presence of increasing numbers of advanced progenitors. Motor neuron generation was also inhibited, albeit less markedly, in explants isolated at stages 15–17 (Figure 5B), a time at which the first postmitotic motor neurons have already been generated. These results indicate that floor plate–derived SHH is required over a prolonged period for the conversion of ventralized progenitors into motor neurons.

Motor Neuron Progenitors Maintain a Dependence on SHH Late into S Phase of Their Final Cell Division

Ventralized progenitor cells lose their dependence on SHH signaling only a few hours before the appearance of the first postmitotic motor neurons (see Figure 5B), and thus probably within their final cell division cycle (Langman et al., 1966). To determine more precisely the stage of the cell cycle at which the SHH dependence of ventralized progenitors is lost, we maintained stage 13 floor plate/ventral explants in vitro in the continued presence of bromodeoxyuridine (BrdU) and analyzed the proportion of motor neurons that had incorporated BrdU after 36 hr. In the presence of H4 IgG, ~40 motor neurons were generated (see Figure 5B). Of these, 83% had not incorporated BrdU (Figures 6B–6E), indicating that the vast majority of SHH-independent motor neuron progenitors had completed their final round of DNA synthesis. The remaining 17% showed low levels of BrdU

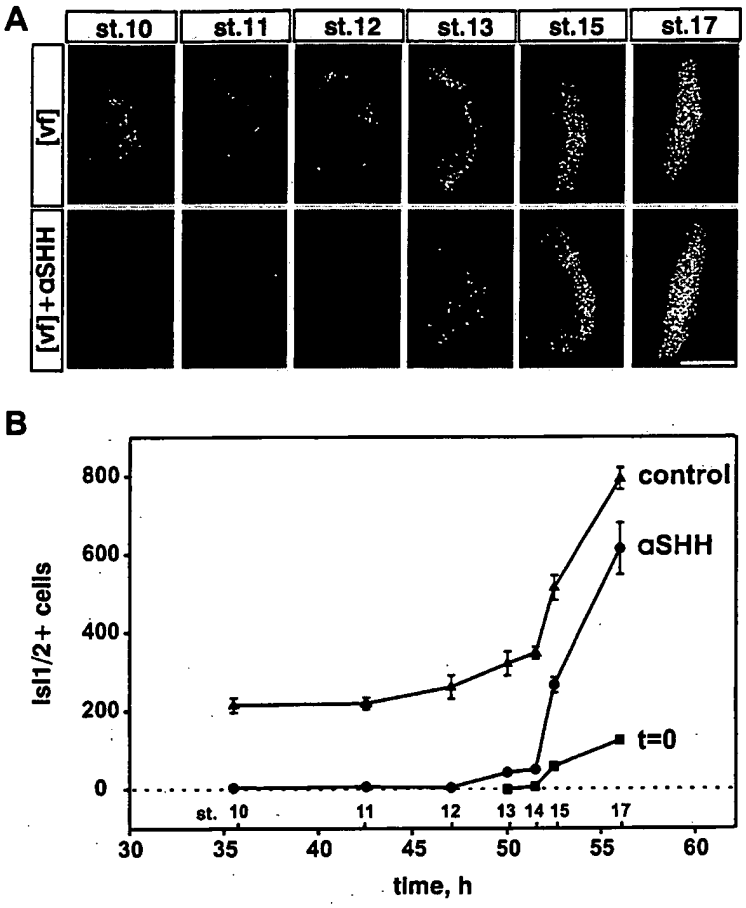

Figure 5. Prolonged Requirement for SHH in the Conversion of Ventral Progenitors into Motor Neurons

(A and B) Isl1/Isl2+ cells in [vf] explants comprising midline and adjacent ventral neural cells grown in vitro for 24–48 hr, in the absence (control) or presence (αSHH) of H4 IgG. (B) shows also the number of Isl1/Isl2$^+$ present in the explant at the time of isolation (t = 0). Values in (B) represent mean + SEM for five explants.

Scale bar in (A), 180 μm.

labeling (Figures 6B–6E), suggesting that these cells were late in their final S phase at the time that they attained independence of SHH signaling. In the absence of H4 IgG, ~300 motor neurons were generated (Figure 5B), of which 86% incorporated BrdU, mostly at high levels (Figures 6B and 6F–6H). The 14% of motor neurons that had failed to incorporate BrdU in explants grown without H4 IgG (Figure 6B) presumably derived from the SHH-independent ventral progenitors revealed in the presence of H4 IgG. Taken together, these results indicate that motor neuron progenitors depend on local SHH signaling until late into their final division cycle.

Ventralized Progenitors Differentiate into Interneurons When SHH Signaling Is Blocked

Blocking the late period of SHH signaling prevents motor neuron generation, raising the issue of the fate of progenitors in this condition. We have considered three possible fates: reversion to a naive state, death, and the adoption of alternative neuronal fates.

Two lines of evidence indicate that ventralized progenitors do not revert to a naive state under conditions in which the late phase of SHH signaling is blocked. First, as described above, PAX7 was not reexpressed in stage 12 ventral explants grown in the presence of anti-SHH antibodies (see Figure 2O). Second, stage 12 ventral or floor plate/ventral explants grown in the presence of both H4 IgG and BMP4 did not give rise to neural crest cells (data not shown), a predicted outcome (Liem et al., 1995) if these cells had reverted to a naive state. Moreover, ventralized progenitor cells appear not to die after blockade of SHH signaling. Incubation of stage 12 floor plate/ventral explants with acridine orange, a marker of pyknotic cells, revealed no difference in the number of labeled cells in the presence and absence of H4 IgG (data not shown).

In view of these results, we determined whether ventralized progenitors might generate interneurons rather than motor neurons under conditions in which the late period of SHH signaling is blocked. To assess interneuron differentiation, we monitored a class of Lim1/Lim2$^+$ interneurons that is generated over the same period as motor neurons in the intermediate region of the neural tube, adjacent to the motor neuron domain (Figure 7K; Tsuchida et al., 1994). Stage 12 floor plate/ventral explants gave rise to many motor neurons and virtually no Lim1/Lim2$^+$ interneurons when grown for 36 hr in vitro (Figures 7A and 7D). In contrast, in the presence of H4 IgG, the blockade of motor neuron differentiation (Figure 7B) was accompanied by the generation of ~150 Lim1/Lim2$^+$ interneurons (Figure 7E). Motor neuron generation in these explants was also blocked by forskolin (Figure 7C), a compound that appears to inhibit the transduction of SHH signals by activating protein kinase A (Fan et al., 1995; Perrimon, 1995), and in this condition also, ~150 Lim1/Lim2 interneurons were generated (Figure 7F). These findings show that ventralized progenitor

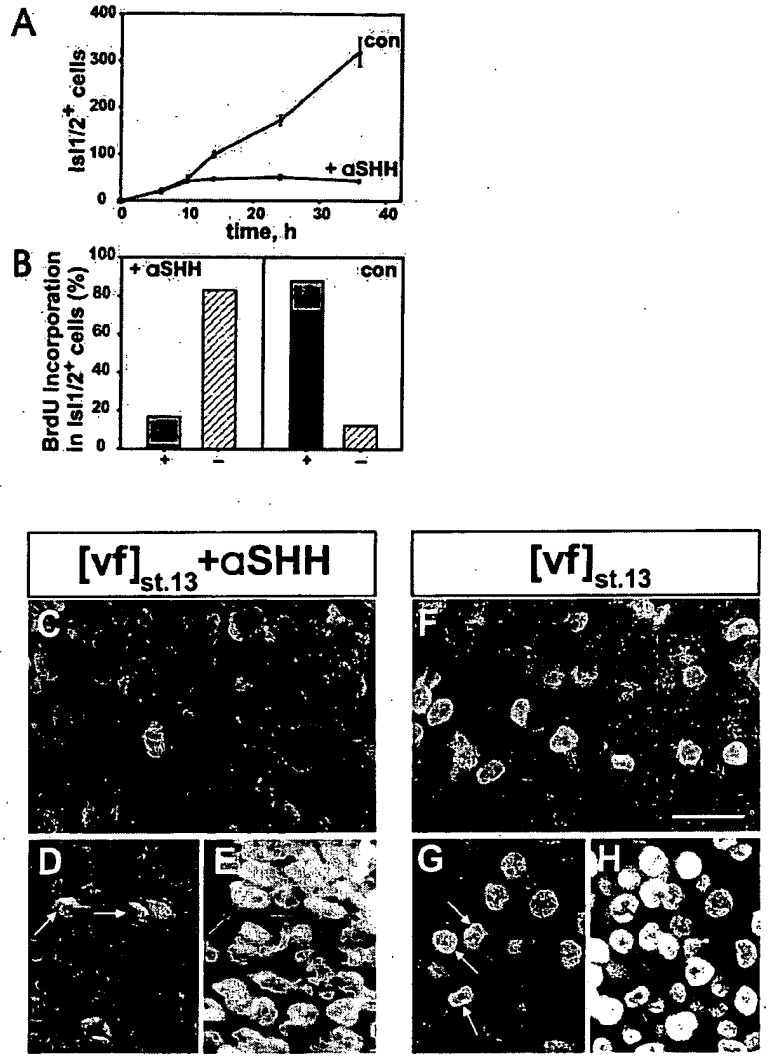

Figure 6. Dependence of Ventralized Progenitors on SHH Signaling Persists Until Late in their Final Cell Cycle

(A) Analysis of Isl1/Isl2 motor neuron generation in stage 13 [vf] explants grown in vitro in the presence (anti-SHH) or absence (control) of H4 IgG.

(B) BrdU incorporation into Isl1/Isl2+ neurons in stage 13 [vf] explants grown in the presence or absence of H4 IgG. Shaded column indicates Isl1/Isl2+ neurons that have incorporated BrdU. Light shading indicates cells that incorporated low levels of BrdU (low is defined subjectively as a labeling intensity below one third that of the most intensely labeled cells). Dark shading indicates cells that incorporated high levels of BrdU. Hatched column indicates Isl1/Isl2+ neurons that did not incorporate BrdU. Histograms are derived from analysis of 155 Isl1/Isl2+ neurons in eight explants grown in H4 IgG and from 318 Isl1/Isl2+ neurons in six control explants.

(C–E) Stage 13 [vf] explants grown in vitro in the presence of BrdU for 36 hr in H4 IgG. BrdU+ cells are green and Isl1/Isl2+ motor neurons red. Of Isl1/Isl2+ neurons, 83% had not incorporated BrdU (C, D). The 17% of Isl1/Isl2+ neurons that were double-labeled incorporated low levels of BrdU (D, E).

(E) BrdU labeling of the field shown in (D) colabeled with anti Isl1/Isl2. Labeling is very weak in motor neurons that have incorporated BrdU (blue arrows).

(F–H) In the absence of H4 IgG, most Isl1/Isl2+ motor neurons had incorporated BrdU (yellow cells) (F, G).

(H) BrdU labeling of the field shown in (G) colabeled with anti-Isl1/Isl2. Virtually all Isl1/Isl2+ motor neurons express high levels of BrdU (blue arrows).

Scale bar, 18 μm.

cells generate Lim1/Lim2 interneurons rather than motor neurons when the late period of SHH signaling is blocked. However, in contrast with motor neurons, the conversion of naive neural plate cells into ventralized progenitors appears not to be a prerequisite for the differentiation of Lim1/Lim2 interneurons, since such neurons were generated (166 ± 27, Lim1/Lim2+ cells, n = 8) in [i] explants grown alone for 36 hr.

These results raise the possibility that a common ventralized progenitor generates motor neurons or interneurons as a function of the ambient SHH concentration. We have not tested this possibility directly. However, one prediction of this idea is that when the SHH concentration is close to the threshold for motor neuron generation, the selection of distinct interneuron or motor neuron fates might be perturbed, with the possible consequence that markers that normally define motor neurons and interneurons might be coexpressed in the same neuron. To test this idea, [i] explants were exposed for 36 hr to 1.2 nM SHH-N, a concentration just above the threshold for induction of motor neurons (Roelink et al., 1995) that resulted in the generation of ~30 Isl1/Isl2+ neurons and ~170 Lim1/Lim2+ interneurons (Figure 7H). Remarkably, 85% of the Isl1/Isl2+ neurons coexpressed Lim1/Lim2 (Figure 7H). Such double-labeled cells were not detected when the SHH-N concentration was increased or decreased ~3-fold (Figures 7G and 7I). Indeed, raising the SHH concentration ~3-fold resulted in the generation of ~300 Isl1/Isl2+ neurons and almost completely suppressed the generation of Lim1/Lim2 interneurons (Figure 7I). Moreover, in [i] explants grown in contact with the notochord, ~5% of Isl1/Isl2 neurons coexpressed Lim1/Lim2, and these cells were located close to the junction of the motor neuron and Lim1/Lim2 interneuron populations (Figure 7J).

These observations prompted us to examine whether such double-labeled neurons could be detected in vivo. Analysis of the neural tube from stages 15 to 19 revealed that ~4% of Isl1/Isl2 neurons coexpressed Lim1/Lim2, but cells of this phenotype were not detected at later stages (Figures 7K and 7L). Strikingly, the cells that

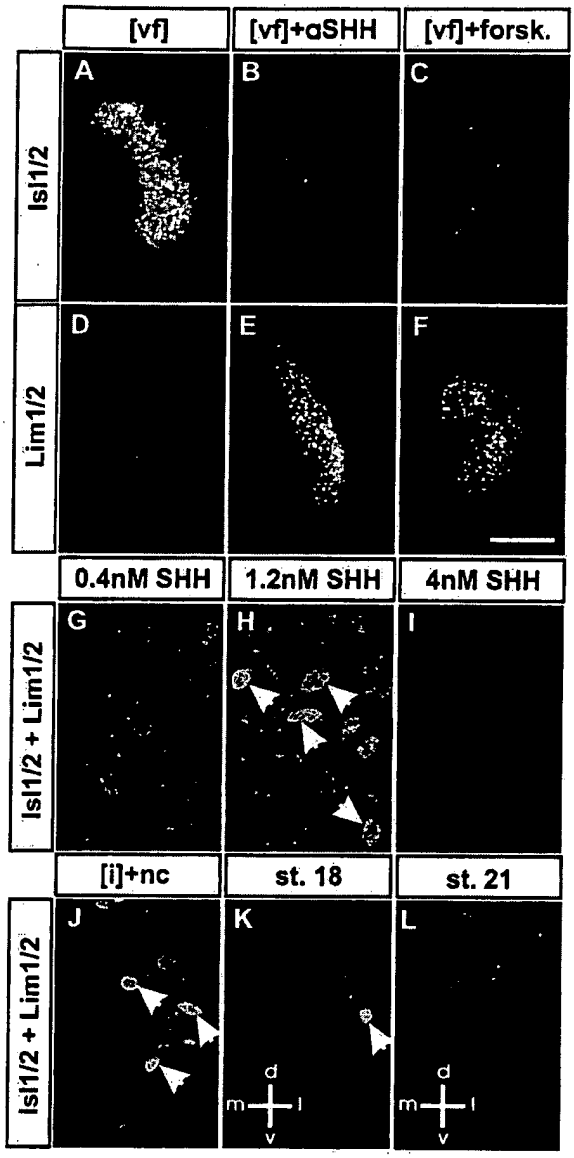

Figure 7. Selection of Motor Neuron and Interneuron Fates by Ventralized Progenitors Is Dependent on Late SHH Signaling

(A and D) Stage 12 [vf] explants grown in vitro for 36 hr generate Isl1/Isl2$^+$ motor neurons (260 ± 30, n = 5; [A]) but few Lim1/Lim2 interneurons (2 ± 1 cells, n = 5; [D]).

(B and E) Stage 12 [vf] explants grown in vitro for 36 hr generate few if any Isl1/Isl2 motor neurons (4 ± 1, n = 5; [C]) but many (158 ± 21, n = 5) Lim1/Lim2 interneurons (E).

(C and F) Stage 12 [vf] explants grown in vitro for 36 hr in forskolin (25 μM) generate few Isl1/Isl2$^+$ motor neurons (7 ± 2, n = 5; [C]) but many (155 ± 16, n = 5) Lim 1/Lim2 interneurons (F).

(G) In the presence of SHH-N (0.4 nM), Lim1/Lim2 neurons (191 ± 30, n = 6) but not Isl1/Isl2 neurons are generated.

(H) In the presence of SHH-N (1.2 nM), both Isl1/Isl2 neurons (28 ± 7, n = 5) and Lim1/Lim2 neurons (171 ± 19; n = 5) are generated, and 85% of the Isl1/Isl2$^+$ neurons (n = 70 cells) coexpress Lim1/Lim2 (arrows).

(I) In the presence of SHH-N (4 nM), many (280 ± 30, n = 5) Isl1/Isl2 neurons and few Lim1/Lim2 neurons (1 ± 1, n = 7) are generated. No double labeled cells are detected.

(J) Coexpression of Lim1/Lim2 is detected in ~5% of Isl1/Isl2$^+$ neurons in [i] explants grown in contact with the notochord for 36 hr. Double-labeled cells are located at the border of the Isl1/Isl2 and Lim1/Lim2 neuronal populations.

(K) Coexpression of Isl1/Isl2 and Lim1/Lim2 (yellow cell, arrow) in stage 18 neural tube. Double labeled cells are located at the border between the motor neuron and interneuron populations and are detected only between stages 15 and 19.

(L) Absence of double-labeled cells at stage 21.

Scale bar in (A)–(F), 150 μm; in (G)–(I), 30 μm; in (J), 40 μm; in (K) and (L), 50 μm.

coexpressed Isl1/Isl2 and Lim1/Lim2 were invariably found at the border of the motor neuron and Lim1/Lim2 interneuron populations (Figure 7K).

These results suggest that the selection of motor neuron and interneuron fates in the ventral neural tube is normally controlled by the ambient SHH concentration at the time of the final division of ventralized progenitor cells. They are also consistent with, but do not prove, the idea that motor neurons and Lim1/Lim2 interneurons can derive from a common ventralized progenitor cell.

Discussion

The present studies establish that SHH-mediated signaling is required for the induction of floor plate differentiation. They also provide evidence that motor neuron generation depends on two critical periods of long-range SHH signaling. During the early period, SHH derived from the notochord is required to convert naive neural plate cells into a ventralized progenitor state that permits the subsequent generation of motor neurons (Figure 8). Such ventralized progenitors, however, are able to generate motor neurons only when exposed to a second period of SHH signaling that persists late into the final division cycle of the progenitor cell. Ventralized progenitor cells generate interneurons rather than motor neurons when this late period of SHH signaling is blocked. Taken together, these results establish a key role for SHH in the induction and patterning of floor plate cells, motor neurons, and interneurons in the ventral neural tube.

The late dependence of motor neuron progenitors on SHH signaling argues against a simple cascade model (Roelink et al., 1995) in which SHH induces the expression of an intermediary diffusible factor that is sufficient to induce motor neurons. If such a factor were to exist, its expression in neuroepithelial cells would have to be dependent on maintained SHH signaling and its activity extremely labile. Our results do not preclude the existence of an intermediary factor that acts in cooperation with SHH to specify motor neuron identity.

Neural Plate Cells Require Early Exposure to SHH for Motor Neuron Generation

Our in vitro studies provide evidence that neural plate cells require exposure to SHH within a critical early period, soon after neural plate formation, in order to maintain their competence to generate motor neurons. The SHH required during this early period derives from the notochord, since SHH expression by cells at the ventral midline of the neural tube is not detected until after

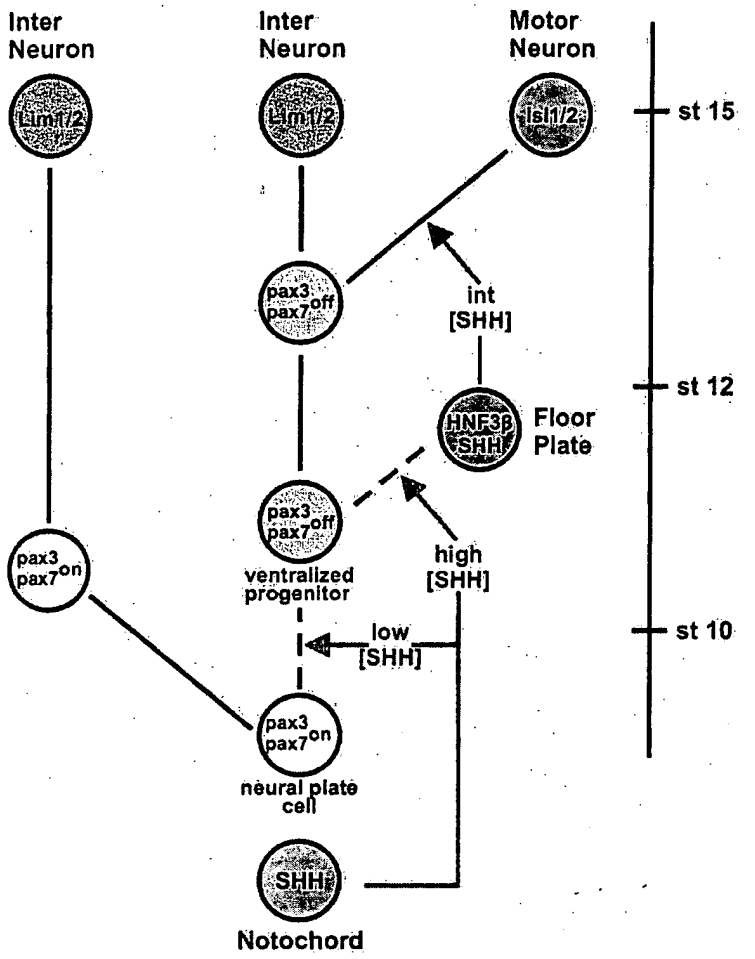

Figure 8. Sequential SHH-Dependent Steps in the Induction of Distinct Cell Types in the Ventral Neural Tube

The model summarizes the cellular source and threshold concentrations of SHH that regulate the differentiation of neural plate cells into floor plate cells, motor neurons, and Lim1/Lim2 interneurons.

SHH derived from the notochord acts at a low concentration threshold (~0.4 nM in vitro) to convert naive neural plate cells into ventralized progenitor cells. This conversion process is marked by the loss of *pax3* and PAX7 expression. Ventralized progenitors at the midline of the neural plate respond to a high local concentration of SHH from the notochord with the generation of floor plate cells (Roelink et al., 1995). The SHH-mediated conversion of naive neural plate cells into floor plate can occur in the absence of cell division (Placzek et al., 1993), as indicated by the broken lines. The generation of motor neurons from ventralized progenitors requires SHH signaling at an intermediate (int) concentration threshold (≥1.2 nM in vitro). The relevant source of SHH for converting ventralized progenitors into motor neurons is likely to be the floor plate. Ventralized progenitors differentiate into Lim1/Lim2 interneurons when the late period of SHH signaling is reduced below the threshold for motor neuron generation. Naive neural plate cells can, however, generate Lim1/Lim2 interneurons without being converted to a ventralized state.

The model depicts a common ventralized progenitor for floor plate cells, motor neurons, and Lim1/Lim2 interneurons. There is evidence that individual neural plate cells differentiate into floor plate cells or motor neurons as a function of the SHH concentration to which they have been exposed (Roelink et al., 1995) but it remains to be established whether motor neurons and Lim1/Lim2 interneurons derive from a common ventralized progenitor cell. The approximate time (HH stages) at which these inductive events occur (at cervical levels) is shown on the right hand side of the figure.

neural cells have acquired a stable ventralized progenitor state.

At present, the only functional indication that neural cells have attained a ventralized state is their ability to generate floor plate cells and motor neurons upon later exposure to SHH. However, the extinction of PAX7 expression by neural plate cells parallels closely their competence to generate ventral cell types, and thus PAX7 appears to provide a valid marker of the conversion of neural plate cells to a ventralized state. The expression of other homeobox genes, notably *pax3*, is also repressed by the notochord and by SHH (Goulding et al., 1993; Liem et al., 1995). Moreover, forced ventral expression of *pax3* in transgenic mice inhibits floor plate differentiation (Tremblay et al., 1996). Taken together, these observations suggest that a critical early step in the SHH-mediated induction of floor plate and motor neuron differentiation is the repression of expression of *pax3* and PAX7.

Ventralized Progenitors Require SHH Signaling to Generate Motor Neurons

The second critical period for SHH signaling in motor neuron generation occurs after naive neural plate cells have been converted into ventralized progenitor cells. Our in vitro studies show that ventralized progenitors deprived of SHH signaling are unable to generate motor neurons. Moreover, BrdU labeling studies indicate that ventralized progenitors maintain their dependence on SHH until late into S phase of the division cycle that precedes the generation of postmitotic motor neurons. The late-dependence on SHH signaling in motor neuron generation sets a constraint on the time at which motor neuron identity is determined, suggesting that it cannot be before late S phase of the final progenitor cell division. These observations are concordant with studies of neurogenesis in the mammalian cerebral cortex, which have shown that the laminar identity of cortical neurons is determined late in the final progenitor cell division cycle (McConnell and Kaznowski, 1991).

Our results suggest that in higher vertebrates the floor plate is the relevant source of SHH required for the conversion of ventralized progenitors into motor neurons. SHH expression is detected in the floor plate by stage 12, and some motor neuron progenitors remain SHH-dependent until at least stage 17, by which time the notochord has been displaced a considerable distance from the ventral neural tube. The late requirement for

SHH signaling in motor neuron generation also suggests a reason, at least in higher vertebrates, for the homeogenetic transfer of SHH expression from the notochord to floor plate cells. In lower vertebrates such as zebrafish, however, the first (primary) motor neurons are generated during gastrulation (Korzh et al., 1993; Kimmel et al., 1994), and the notochord retains contact with the ventral neural tube for a prolonged period. Thus, SHH derived from the axial mesoderm may be sufficient to generate motor neurons in lower vertebrates. Consistent with this idea, motor neurons are present in zebrafish embryos carrying the *cyclops* mutation (Hattä et al., 1991; Hattä, 1992), in which *HH* genes are not expressed at the midline of the neural plate (Krauss et al., 1993; Ekker et al., 1995).

Motor Neuron Generation Requires Long-Range SHH Signaling from the Notochord and Floor Plate

The present studies provide evidence that SHH signaling operates at a distance during both the early and late critical periods. During the early period, the domain of the neural plate over which PAX7 expression is repressed extends ~10 cell diameters from the notochord, a distance that may indicate the range of SHH action. In support of this, an elevated level of *patched* mRNA, diagnostic of exposure of cells to HH signaling in flies and vertebrates (Perrimon, 1995; Goodrich et al., 1996), is detected in the medial neural plate and ventral neural tube in a domain similar to that in which PAX7 expression is repressed (Goodrich et al., 1996; Marigo and Tabin, 1996).

Similarly, the late conversion of ventral progenitors into motor neurons appears to depend on long-range SHH signaling from the floor plate. Ventral neural tube explants isolated from floor plate cells retain a dependence on local SHH signaling for the generation of motor neurons. Since *Shh* mRNA is not expressed in ventral explants, the SHH protein present in explants appears to have derived by diffusion from a midline source, presumably the floor plate. Diffusible forms of SHH derived initially from the notochord and later from the floor plate are likely therefore to act at a distance to promote the generation of motor neurons.

SHH Signaling Directs Neuronal Fate

The state of SHH signaling during the final division of ventralized progenitor cells appears to determine whether motor neurons or interneurons are generated. Under conditions of SHH signaling, ventralized progenitors differentiate into motor neurons, but when SHH signaling is blocked, Lim1/Lim2 interneurons rather than motor neurons are generated. In vitro, ventralized progenitors appear to generate these two distinct neuronal cell types in response to changes in the SHH-N concentration of only ~3-fold. The establishment of distinct dorsoventral domains of the neural tube within which motor neurons and Lim1/Lim2 interneurons are generated is likely therefore to be controlled by the spatial extent of SHH signaling immediately prior to the onset of neuronal differentiation.

Our results have not resolved whether the SHH-dependent selection of distinct neuronal fates is exerted at the level of a common ventralized progenitor cell. Lineage tracing studies in the chick neural tube have shown that ventral progenitor cells frequently generate clones that include motor neurons and interneurons (Leber et al., 1990). Moreover, the coexpression by single neurons of LIM homeodomain protein markers of motor neuron and interneuron fates when the SHH concentration is at the threshold for motor neuron generation can most easily be explained if these distinct neuronal fates are normally available to an individual ventralized progenitor cell. In vivo, such double-positive cells are detected transiently at the junction of the motor neuron and interneuron populations, a position at which the local SHH concentration is likely to be close to the threshold for motor neuron generation.

Whether the Lim1/Lim2 interneurons that are generated in the intermediate region of the neural tube derive exclusively from ventralized progenitor cells remains unclear. Lim1/Lim2 interneurons can derive from ventralized progenitors if the late period of SHH signaling is blocked but can also be generated from naive neural plate cells that have not been exposed to SHH. Thus, the SHH-mediated conversion of naive neural plate cells to a ventralized progenitor state may not be a prerequisite for the generation of Lim1/Lim2 interneurons (Figure 8). Alternatively, the interneurons generated from naive and ventralized progenitors may represent distinct neuronal classes that share expression of Lim1/Lim2.

Finally, the present studies show that the SHH-N concentration threshold required to maintain the competence of neural plate cells for motor neuron generation is ~3-fold lower than the concentration threshold required later for the acquisition of motor neuron fate by ventralized progenitors. In turn, the SHH-N concentration threshold for induction of floor plate differentiation is ~3-fold greater than that for motor neuron generation (Roelink et al., 1995). Taken together, these findings suggest that different SHH-N concentration thresholds operate at sequential periods during the generation of a single neuronal cell type (Figure 8). They also support the idea that in vivo, SHH controls the identity and pattern of cell types generated in the ventral neural tube through actions at multiple concentration thresholds.

Experimental Procedures

Generation of Anti-SHH-N Antibodies

A cDNA clone encoding SHH-N (Porter et al., 1995) (residues 1–198 of rat SHH; Roelink et al., 1994) was cloned into a baculovirus expression vector (Invitrogen), and SHH-N protein derived from Tn-5B1–4 cells (Wickham et al., 1992) was purified (Porter et al., 1995). Monoclonal anti-SHH antibody 5E1 (IgG1 isotype) was obtained by fusion of spleen cells from SHH-N-immunized mice with NS1 myeloma cells. IgG fractions were obtained by affinity purification on protein A–agarose. H4 IgG fraction was used at a final concentration of 250 μg/ml, SHH-N affinity-purified H4 IgG at 50 μg/ml, and protein A–purified 5E1 IgG at 2.5 μg/ml, with similar results.

SHH-N Protein

SHH-N protein was purified as described above. The SHH-N concentration was determined by amino acid analysis after separation of the ~20 kDa SHH-N protein by SDS–polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose membranes. Protein concentrations indicated are based on a transfer efficiency of 100%. Average protein recovery after transfer, determined by comparison with Escherichia coli–derived mouse SHH-N (provided

by P. Beachy) is ≥50%; thus, the actual SHH-N protein concentration used in assays may be greater by a factor of ≤2.

Immunocytochemistry

Immunocytochemical localization of proteins was performed at the level of the twelfth somite as described (Yamada et al., 1991). SHH was detected with MAb 5E1, HNF3β with MAb 4C7 anti-chick HNF3β (A. Ruiz i Altaba, S. M., and T. J., unpublished data), PAX7 (Jostes et al., 1990) with a monoclonal anti-chick PAX7 antibody that does not react with PAX3 (A. K., unpublished data), Isl1/Isl2 with MAb 4D5 and rabbit antibody K5 (Tsuchida et al., 1994), and Lim1/Lim2 with MAb 4F2 and rabbit antibody T2 (Tsuchida et al., 1994). Coexpression of proteins was detected by using a Bio-Rad 600 confocal microscope.

Neural Plate Explant Assay

Neural plate, neural tube, and notochord tissue from Hamburger and Hamilton (1951) (HH) stage 10–17 embryos was isolated from the 12-somite level, independent of stage, and grown in vitro (Yamada et al., 1993). BMP4 protein was obtained as described (Liem et al., 1995).

BrdU Incorporation Studies

BrdU incorporation into neural plate cells was analyzed by labeling explants with 170 nM BrdU. Double-labeling experiments were performed with MAb 4D5 and Cy3-conjugated goat anti-mouse IgG (Jackson Labs) and FITC-conjugated anti-BrdU (Becton Dickinson) and analyzed on a Bio-Rad 600 confocal microscope.

RT–PCR Assay v

Analysis of *Isl1*, *Shh*, and *S17* expression was performed as described (Tanabe et al., 1995).

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Note Added in Proof

Analysis of the neural phenotype of mice lacking *Sonic Hedgehog* function has also provided evidence that SHH is required to initiate cell patterning in the ventral neural tube: Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic Hedgehog* gene function. Nature *383*, 407–413.

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Mapping Sonic Hedgehog-Receptor Interactions by Steric Interference*

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We have defined regions in the Sonic hedgehog (Shh) molecule that are important for Patched (Ptc) receptor binding by targeting selected surface amino acid residues with probes of diverse sizes and shapes and assessing the effects of these modifications on function. Eleven amino acid residues that surround the surface of the protein were chosen for these studies and mutated to cysteine residues. These cysteines were then selectively modified with thiol-specific probes, and the modified proteins were tested for hedgehog receptor binding activity and their ability to induce differentiation of C3H10T1/2 cells into osteoblasts. Based on these analyses, approximately one-third of the Shh surface can be modified without effect on function regardless of the size of the attachment. These sites are located near to where the C terminus protrudes from the surface of the protein. All other sites were sensitive to modification, indicating that the interaction of Shh with its primary receptor Ptc is mediated over a large surface of the Shh protein. For sites Asn-50 and Ser-156, function was lost with the smallest of the probes tested, indicating that these residues are in close proximity to the Ptc-binding site. The epitope for the neutralizing mAb 5E1 mapped to a close but distinct region of the structure. The structure-activity data provide a unique view of the interactions between Shh and Ptc that is not readily attainable by conventional mapping strategies.

Hedgehog proteins are a highly conserved family of extracellular signaling molecules with fundamental roles in embryonic development both in vertebrates and in invertebrates (for reviews see Refs. 1–4). In mammals, three homologs have been identified that are referred to as Sonic, Indian, and Desert hedgehog. Of these forms, Sonic hedgehog (Shh)[1] has been the most extensively characterized. Shh is involved in diverse embryonic events, including the induction of floor plate, the establishment of ventral polarity within the central nervous system, and proper anterior-posterior patterning of the developing limbs (5–8). In mediating these effects, Shh is believed to act both as a short range, contact-dependent inducer and as a long range morphogen (2, 7–11). It is unclear whether the same forms of the protein contribute to both modes of action. The importance of Shh for correct embryonic development is highlighted by the severity of malformations associated with human mutations in the *Shh* gene (12) and by the developmental defects observed in the Shh knockout mouse leading ultimately to its embryonic lethal phenotype (13).

Shh is synthesized as a 45-kDa precursor protein that is cleaved autocatalytically to yield a 20-kDa N-terminal fragment (residues 24–197 in the human gene sequence) that is responsible for all known hedgehog biological activity and a 25-kDa C-terminal fragment that contains the auto-processing machinery (14–16). The N-terminal fragment remains membrane-associated through the addition of two lipid tethers, a palmitic acid at its N terminus (17) and a cholesterol at its C terminus (18, 19). *In vivo*, the lipid tethers restrict the tissue localization of the Hedgehog signal and presumably have evolved as part of the mechanism for regulating short range-long range signaling. Whereas both modifications are likely to be important for tethering Shh to the plasma membrane, lipid modifications on the N terminus result in about a 30-fold increase in potency (17). How the N-terminal modification regulates potency is unknown. The three-dimensional crystal structure of a fragment of murine Shh (residues 39–195) has been solved (20). This information has proved to be invaluable for probing structure-function relationships of the protein.

Although the mechanism of action of hedgehog proteins is not fully understood, biochemical and genetic data suggest that the receptor for Shh is the product of the tumor suppressor gene *patched* (*ptc*) (21–23) and that other proteins, including *smoothened* (*smo*) (23, 24), *Cubitus interruptus*, or its mammalian counterpart *gli* (25, 26) and *fused* (27), are involved in the hedgehog signaling pathway. Homology between the *ptc* sequence and other gene products suggests that it is a member of the 12 transmembrane family of proteins. Although Ptc is the receptor for Shh, it also acts as a negative modulator of the Shh signal. In the absence of Shh, Ptc represses signaling through Smo. Shh binding to Ptc causes derepression of Smo, allowing signaling to occur. Subsequent induction of Ptc may act to turn off hedgehog (Hh) signaling. From the analysis of *smo* null mutants in *Drosophila*, Ptc appears to bind Hh without the participation of Smo (28). Other Hh-binding proteins, including a *ptc* homolog, *ptc-2* (23), and hedgehog-interacting protein (29) have been identified, raising the potential for additional levels of regulation. In order to study the interactions of Shh with Ptc, we developed a mapping strategy where selected amino acid residues on the surface of Shh were mutated into cysteine residues, and after modification of these cysteines with probes of different size and shape, we determined which of the modifications disrupted function. The structure-activity data provide a unique view of Shh-Ptc interactions. This approach has significant advantages over most conventional mapping strategies and should be widely applicable to most other proteins, particularly those for which a structure is available.

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[1] The abbreviations used are: Shh, Sonic hedgehog; Ptc, Patched; Hh, hedgehog; MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol; BzM, benzophenone-4-maleimide; NEM, *N*-ethylmaleimide; AP, alkaline phosphatase; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

Exhibit 2

EXPERIMENTAL PROCEDURES

Construction of Shh Mutants—Shh (residues 24–197 in the human sequence) and Shh mutants were expressed in *Escherichia coli* as His-tagged fusion proteins as described previously (17, 30). Point mutations were incorporated into the *Shh* DNA by unique site elimination mutagenesis using an Amersham Pharmacia Biotech kit following the manufacturer's recommended protocol. Mutagenic oligonucleotides were designed so that the incorporation of each mutation also resulted in the addition or removal of a specific restriction enzyme site. The following mutagenic primers were used, and the resulting restriction sites that were added as a result of the mutagenesis steps are listed below: 1) A192C, 5′ GAG TCA TCA GCC TCC CGA TTT TGC GCA CAC CGA GTT CTC TGC TTT CAC C 3′, which introduces a new *Fspl* site; 2) N50C, 5′ GGC GCC TAG GGT CTT CTC AGC CAC ACA GGG GAT AAA CTG CTT GTA GGC 3′, which introduces a new *Dde*I site; 3) N69C, 5′ GAGTTC CTT AAA TCG CTC GGA GCA CCT GGA GAT CTT CCC TTC 3′, which introduces a new *Bsp*1286I site; 4) Y80C, 5′ CAT CCT TAA ATA TGA TGT CCG GGT TGC AAT TGG GGG TGA GTT CCT TAA ATC G 3′, which introduces a new *Nci*I site; 5) N91C, 5′ CAT CAG CCT GTC CGC TCC GGT ACA TTC TTC ATC CTT AAA TAT GAT GTC 3′, which introduces a new *Rsa*I site; 6) K105C, 5′ GAG ATG GCC AAA GCG TTT AAG CAG TCC TTA CAC CTC TGA GTC 3′, which introduces a new *Mse*I site; 7) N115C, 5′ GTT TCA CTC CTG GCC ACT GAC ACA TCA CCG AGA TGG CCA AAG 3′, which removes a *Bsr*I site; 8) S135C, 5′ GTA GTG CAG AGA CTC CTC GCA GTG GTG GCC ATC TTC G 3′, which removes a *Dde*I site; 9) S156C, 5′ CCA GCA TGC CGT ACT TGC AGC GAT CGC GGT CAG ACG TGG 3′, which introduces a new *Pvu*I site; 10) S177C, 5′ CAG TGG ATA TGT GCC TTG CAC TCG TAG TAC ACC CAG TC 3′, which removes a *Hin*fI site; and 11) G169C, 5′ GTA CAC CCA GTC GAA GCA GGC CTC CAC CGC CAG GC 3′, which removes an *Msp*I site. The fidelity of the resulting constructs was verified by DNA sequencing. Expression vectors were generated by subcloning the DNA inserts into phosphatase-treated, 5.64-kilobase pair *Xho*I-*Nco*I pET11d vector backbone of p6H-Shh (30), which encodes a 6-histidine tag and an enterokinase cleavage site engineered immediately adjacent to the start of the mature Shh sequence. The presence of the introduced restriction site change was reconfirmed in the expression vector. The expression vectors were transformed into competent *E. coli* BL21(DE3)pLysS, colonies were selected, induced with isopropyl-1-thio-β-D-galactopyranoside, and screened for Shh expression as described previously (17, 30). *Shh* DNA containing C24II and A192C mutations was used as the template for mutagenesis steps 2–12. The C24II framework, in which cysteine 24 was replaced with two isoleucine residues, was selected for these studies since we had determined that modifications on Cys-24 would otherwise compromise the mapping analysis. The C24II substitution not only prevented the formation of undesired modification products but generated a form of the protein that was 10 times as active as wild type Shh in the C3H10T1/2 differentiation assay.[2]

Purification and Analysis of Shh Mutants—The mutant Shh proteins were purified following the strategy previously described for the purification of wild type Shh (30). Bacteria expressing the mutants were lysed in a French press, and particulates were removed by centrifugation. The clarified lysates were subjected to sequential chromatography on nickel-nitrilotriacetic acid-agarose (Qiagen) and SP-Sepharose (Amersham Pharmacia Biotech). The resulting proteins were treated with enterokinase to remove the His tag and repurified on nickel-nitrilotriacetic acid-agarose and SP-Sepharose columns. The purified proteins were aliquoted and stored at −70 °C.

The following thiol reactive reagents were used for structure-activity studies: *N*-ethylmaleimide (NEM) and β-(4-hydroxyphenyl)ethylmaleimide were from Pierce; *N*-(1-naphthyl)maleimide was from Aldrich; *N*-(1-pyrenyl)maleimide was from Sigma; benzophenone-4-maleimide (BzM) was from Molecular Probes; PEG 5000 maleimide was from Fluka; and PEG 20,000 maleimide was from Shearwater Polymers, Inc. Working stock solutions of NEM and PEG were dissolved in water, BzM in dimethylformamide, and the other reagents in dimethyl sulfoxide. For modification with thiol reactive probes, Shh proteins at 2.3 mg of Shh/ml in 50 mM MES, pH 6.5, 230 mM NaCl, 0.06 mM DTT were treated for 2 h at room temperature with a 1.1–2-fold molar excess of each thiol-reactive compound over the total concentration of free thiol groups in the reaction mixtures. Samples were then treated with 0.5 mM DTT for 1 h at room temperature to quench excess modification reagent and analyzed for activity in the C3H10T1/2 and Ptc binding assays.

[2] F. R. Taylor and E. A. Garber, unpublished data.

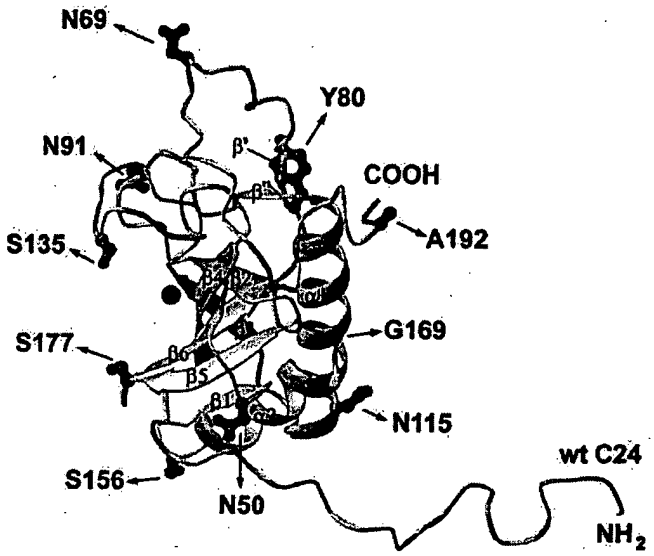


FIG. 1. **Selection of mutagenesis targets.** The crystal structure of Shh was scanned for potential sites where modifications could serve as probes for function. The positions of these sites are shown with respect to a hybrid model containing residues 24–188 from the human Shh C24II crystal structure[3] and residues 189–194 from the murine Shh crystal structure (residues 190–195 using the murine numbering system) (20). The human and mouse sequences differ by only one amino acid residue, and the structures are highly homologous. α-Helices and β-sheets are numbered following the nomenclature previously defined (20). The *solid circle* at the *left* of the model shows the position of a Zn^{2+} atom in the structure. Side chains of target residues are enlarged to enhance visualization but reflect their actual orientation in the human and murine crystal structures.

For cross-linking studies with BzM, samples of Shh at 1.1 mg/ml in 50 mM MES, pH 6.5, 230 mM NaCl, 0.13 mM DTT, 0.1% Tween 80 were treated with a stoichiometric amount of cross-linker (0.5 mM diluted from a 25 mM stock solution). The preformed Shh-BzM conjugates were incubated with Ptc-transfected embryonic kidney 293 cells (30) for 1 h at room temperature and subjected to cross-linking and analysis as described previously (31). Western blots were probed first with rabbit anti-Shh antibody and then with affinity purified rabbit anti-Ptc antibody (30).

Ptc binding and C3H10T1/2 AP induction assays were performed as described previously (30). For Ptc binding experiments, EBNA 293 cells transiently transfected with a myc-tagged, C-terminally truncated murine *ptc* construct (22) (a gift of Matt Scott, Stanford University) were titrated with serial 2-fold dilutions of each test compound in the presence of 5 nM of the reporter. Cells were washed, fixed, and read on a fluorescence-activated cell sorter. Binding constants were calculated from single determinations for each sample. For assessing activity, C3H10T1/2 cells (American Type Culture Collection) were incubated for 5 days with serial dilutions of each Shh preparation. The cells were lysed and assayed for alkaline phosphatase (AP) activity using the chromogenic substrate *p*-nitrophenyl phosphate and read at 405 nm. Each sample was analyzed in duplicate, and EC_{50} values were measured from the mean data values.

RESULTS AND DISCUSSION

Production and Characterization of Shh Surface Mutants—We have used the x-ray structure of Shh to identify potential surface residues that could serve as targets for modification. Eleven sites were selected for these studies. Most of these sites lie on loops where minimal contacts occur between the side chains of the selected residues and other parts of the molecule, and together they cover the entire surface of the molecule. The positions of the sites with respect to the Shh structure are summarized in Fig. 1. These amino acids were mutated to cysteine residues by unique site elimination mutagenesis, and the mutagenized genes were expressed in *E.*

[3] P. A. Boriack-Sjodin and E. A. Garber, unpublished results.

TABLE I
Analysis of Shh Cys mutants for Ptc binding and activity on C3H10T1/2 cells

Hedgehog proteins that had been treated with PEG 5000 maleimide, NEM, or left unmodified were evaluated for Ptc binding activity on EBNA 293 cells transfected with a truncated form of the gene for Ptc and for their ability to induce the differentiation of C3H10T1/2 cells.

Protein, Shh variant	EC_{50} in C3H10T1/2 assay in μg/ml			K_d for Ptc binding in ng/ml ($\times 10^{-1}$)		
	Unmodified	PEG 5000	NEM	Unmodified	PEG 5000	NEM
Wild type	2	>20	1	4	84	2
C24II/A192C	0.3	0.3	0.2	2	2	1
+N50C	0.4	4	0.3	2	64	1
+N69C	0.4	0.3	0.2	4	4	4
+Y80C	0.2	0.2	0.2	6	6	2
+N91C	0.3	0.2	0.2	6	6	4
+K105C[a]	0.2	—	—	6	—	—
+N115C	1.5	>20	2	8	70	1
+S135C	0.4	0.6	0.2	8	16	1
+S156C	0.4	2	0.2	8	66	1
+G169C	0.3	0.5	0.3	4	60	4
+S177C	1	5	2	8	170	16

[a] Modification chemistries failed.

coli. The proteins were purified and tested for their ability to bind to Ptc. All 11 of the mutants exhibited wild type Ptc binding activity with IC_{50} values ranging from 20 to 80 ng/ml (1–4 nM) (see Table I). The binding data indicated that the mutagenesis had little functional impact on the resulting proteins and that none of the selected residues were critical for Ptc binding.

The 11 mutants were also tested for their ability to induce differentiation of C3H10T1/2 cells into osteoblasts. We and others (17, 30, 32) have observed that C3H10T1/2 cells are Shh-responsive and that hedgehog activity on this line can be readily assessed by measuring the induction of AP, a marker of mature differentiated osteoblasts. AP induction on C3H10T1/2 cells provides a simple, quantitative measure of the *in vitro* potency of Shh without the complication of having to work with primary cell cultures or organ explants. All of the mutants were functional in the C3H10T1/2 assay (Table I). Nine of the eleven mutants exhibited activity equivalent to the C24II Shh parent molecule, with EC_{50} values ranging from 0.2 to 0.4 μg/ml. The two other mutants, N115C and S177C, showed about a 3–5-fold loss of potency.

To test the accessibility of the engineered cysteines to modification, the protein preparations were treated with PEG 5000 maleimide, and the reaction products were analyzed by SDS-PAGE (Fig. 2). Because of hydration of the PEG in aqueous buffer, addition of a PEG 5000 moiety should produce a shift in mass of about 10–15 kDa, and thus a singly pegylated Shh product should have an apparent molecular mass of 30–35 kDa and a doubly modified product, a molecular mass of about 45 kDa. All but mutant K105C showed the predicted pegylation products. The 20–25-kDa shift in the masses after pegylation of mutants N50C, N69C, Y80C, N91C, N115C, S135C, S156C, and S177C is consistent with the presence of two potential sites for modification (see Fig. 2*B*). The slight variations in their electrophoretic mobilities are probably due to differences in shape that result from PEG attachment at the different positions in the Shh sequence. Previously we reported (33) an analogous effect for protein-protein complexes that had been cross-linked at different positions along their sequences. The 12-kDa shift in mass after pegylation of the C24II/A192C variant, the parent that was used as a template for mutagenesis, is consistent with the presence of a single site for modification. As further verification that the pegylated products contained the expected number of PEGs, selected products were subjected to mass spectrometry on a matrix-assisted laser desorption/

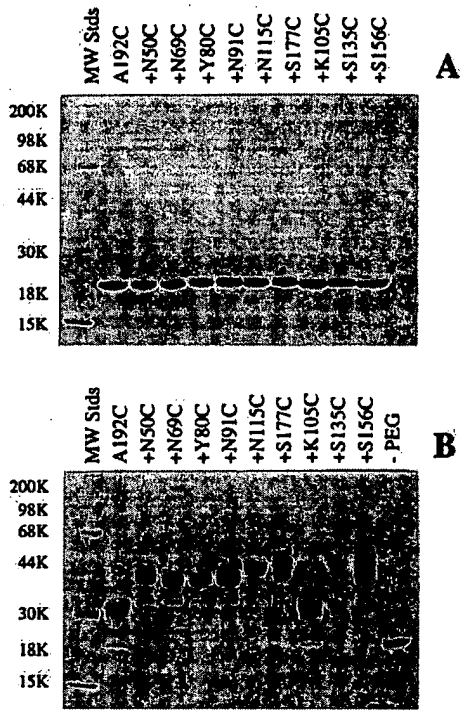


FIG. 2. **Analysis of pegylated Shh by SDS-PAGE.** Shh mutants (4 μg/lane) were subjected to SDS-PAGE on a 10–20% gradient gel (Daiichi) (*A*) without pegylation and (*B*) after treatment with 5000 PEG maleimide. Proteins were stained with Coomassie Brilliant Blue. Lane designations correspond to the individual mutant analyzed. The −PEG designation in *B* is Shh C24II/A197C that had not been treated with PEG. *MW Stds* are prestained high molecular weight markers. Apparent masses of the molecular weight standards are indicated at the *left*.

ionization-time of flight mass spectrometer. Masses consistent with the predicted number of modifications were observed (data not shown).

In addition to the engineered cysteines, the C24II Shh sequence contains two other free cysteines at residues 102 and 187 that are not accessible for modification. Based on the Shh crystal structure, neither of the sites is surface-exposed, and therefore they were not expected to react with the modification agents. Peptide mapping (17) was used to verify the absence of modification of these sites without prior denaturation of the protein (data not shown). Even with NEM, the smallest of the modification reagents tested in this study, the modification reactions were highly specific for the free thiols on the engineered cysteines.

Typically we observe that modification reactions with maleimides go to about 95% completion. As shown in Fig. 2*B*, no unreacted Shh remained after treatment of the mutants with the PEG 5000 maleimide, but about 5% of the product contained a single modification as evidenced by the presence of the minor band at 32–35 kDa. Throughout the studies described below, assays were performed on products that were treated with the maleimide probes and quenched to prevent further reaction but that had not been further purified. Consequently, a complete blockage of function after modification would be expected to decrease binding or potency by about 20-fold. Our inability to modify cysteine 105 in the K105C mutant was probably due to inadequate exposure of the thiol group on the engineered cysteine. After denaturation and subsequent reaction with PEG, we observed a shift in mass consistent with modification at this site (data not shown).

Mapping Shh-Receptor Interactions by Steric Interference—The pegylated mutants were tested for function in the Ptc binding and C3H10T1/2 assays. Mutants N69C, Y80C, and

N91C were fully active in both assays; mutants S135C and G169C were slightly reduced in their activity in the C3H10T1/2 assay but then exhibited a more significant loss of potency for Ptc binding; and mutants N50C, N115C, S156C, and S177C were greatly reduced in activity both in their ability to bind Ptc and to induce AP expression (Table I). Modification of wild type Shh through the N-terminal cysteine with PEG 5000 maleimide also resulted in a large reduction in the activity of the protein in both assays (Table I). To control for possible effects of the maleimide chemistry on function, samples were also treated with N-ethylmaleimide (125 Da) and assayed for Ptc binding and the induction of AP on C3H10T1/2 cells (Table I). NEM treatment either had no effect or in most instances resulted in a slight increase in activity. Because NEM treatment was highly selective for the engineered cysteines and had no deleterious effect on activity, we selected the maleimide chemistry as the basis for all subsequent modification work.

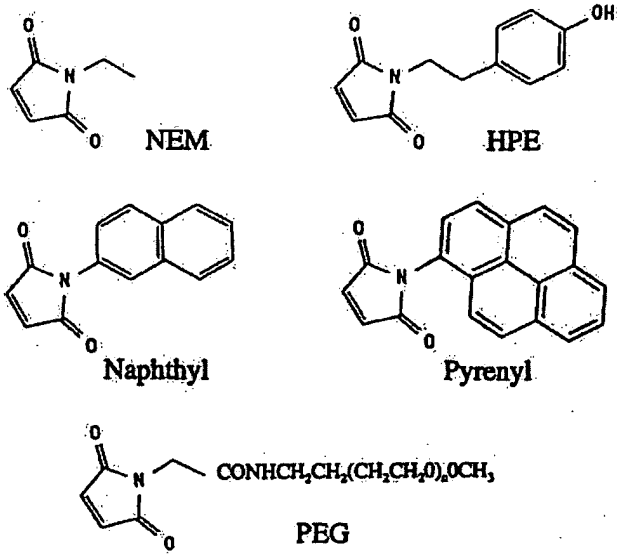
When the activity data for the pegylated compounds were analyzed with respect to the position of the mutations in the crystal structure of Shh, the data revealed regions in the protein that were required for function. The engineered cysteines from mutants N69C, Y80C, and N91C for which pegylation had no effect on function all resided on the same surface of the protein from which the C terminus of the protein protrudes. Since a cholesterol is attached at or near the C terminus of Shh in its natural form (18, 19), this region is likely to be associated with the cell membrane and would not be expected to be involved in receptor binding. Since pegylation of all other mutants affected function, albeit to varying degrees, little additional information could be extracted from the data. In the crystal structures of mouse and human Shh, a Zn^{2+} ion is coordinated to the protein in an arrangement similar to that seen in zinc hydrolases such as carboxypeptidase A. It was interesting that mutants S135C and S177C, which were designed to incorporate PEG maleimides on either side of the cleft containing the metal-binding site, retained partial activity. If Shh activity required the metal-binding site to interact directly with another protein, then PEG modification at those sites should have totally inactivated the protein. In recent studies, where mutagenesis was used to eliminate key amino acids that would be needed for enzymatic activity if the Zn^{2+} had a catalytic role, the mutations were without effect, further indicating that catalysis is not required for hedgehog function (35, 36).

Refinement of the Structure-Activity Data Using Alternative Probes—Because the PEG 5000 modification divided the mutants into three groups (active, inactive, and partially active) and NEM alone had no clear effect on function, we reasoned that it would be possible to refine further the analysis by selecting and analyzing the effects of modifying groups of other sizes and shapes. Maleimide-containing probes are the most useful for this type of analysis because there are over 100 different commercially available *N*-substituted maleimide reagents of varying sizes and shapes. Four additional maleimide probes were selected, three smaller versions containing either a single aromatic ring (hydroxyphenyl), three aromatic rings (naphthyl), or four rings (pyrenyl), and a larger form containing a PEG 20,000. Results from these analyses are summarized in Table II. When the Shh mutants were modified with PEG 20,000, the same three mutants (N69C, Y80C, and N91C) that retained function with PEG 5000 remained active after derivatization with the PEG 20,000, whereas mutants S135C and G169C, which were partially inhibited by the PEG 5000, exhibited greater inhibition by modification with the PEG 20,000 PEG. The other mutants that were inactive after modification with PEG 5000 were also inactive after modification with 20,000 PEG.

TABLE II

The effects of thiol modifications on AP induction

Hedgehog proteins were treated with each thiol reactive compound indicated below and analyzed for activity in the C3H10T1/2 assay.



Shh variant	Unmodified	NEM	HPE	Naphth	Pyrene	PEG 5000	PEG 20,000
C24II/A192C	0.3	0.2	0.2	0.2	0.2	0.3	0.3
+N50C	0.4	0.3	0.5	1.5	3	4	8
+N69C	0.4	0.2	0.4	0.4	0.5	0.3	0.3
+Y80C	0.2	0.2	0.3	0.2	0.3	0.2	0.3
+N91C	0.3	0.2	0.2	0.3	0.3	0.2	0.4
+N115C	1.5	2	2	4	3	>20	>20
+S135C	0.4	0.2	0.2	0.3	0.3	0.6	2
+S156C	0.4	0.2	0.2	0.6	3	2	2
+G169C	0.3	0.3	0.2	0.2	0.3	0.5	2
+S177C	1	2	2	2	3	5	8

When the Shh mutants were treated with the smaller maleimide probes and tested for function, we observed a clear dependence of the size and shape of the modifying group on its ability to interfere with function. Of the six sites where modification with 5000 PEG impacted function (Cys-24, Asn-50, Asn-115, Ser-177, Ser-135, and Ser-156), only two, Asn-50 and Ser-156, were affected by the addition of the pyrenyl moiety. Pyrene maleimide treatment resulted in a 10-fold loss in the potency of the N50C mutant and a 15-fold loss for the S156C mutant as compared with the NEM-treated controls. The activity of mutant N50C was also reduced by 5-fold following modification with the naphthyl moiety and by 1.7-fold after addition of the hydroxyphenyl moiety, whereas the activity of mutant S156C was reduced by 3-fold by the naphthyl group and was not affected by the hydroxyphenyl moiety. Thus by use of this steric interference analysis, it was possible to distinguish between the mutants by the size of the modifying group needed to block function.

Mapping Shh-Ptc Interactions by Cross-linking—Shh-Ptc interactions were also evaluated using cross-linking to probe the proximity of the mutated sites to Ptc (Fig. 3). For these analyses, the Shh mutants were first modified with the photoreactive cross-linker benzophenone-4-maleimide. Ptc-transfected 293 cells were incubated with the BzM-activated Shh conjugates, exposed to UV light, and lysates from the cells subjected to Western blotting. The blots were probed first with an anti-Shh antibody and then with an anti-Ptc antibody. When the blots were probed for Ptc expression (Fig. 3*A*), three bands were observed with masses of approximately 80, 170, and 270 kDa. These bands were not detected in lysates from untransfected

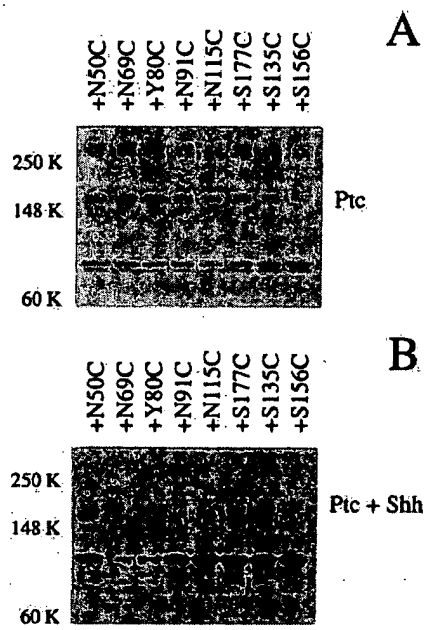


FIG. 3. **Analysis of Shh-Ptc interactions by cross-linking.** Ptc-transfected 293 cells were incubated with the benzophenone maleimide conjugate of each of the Shh mutants indicated and subjected to photo cross-linking. Cell lysates were subjected to SDS-PAGE/Western blotting and probed with affinity purified anti-Ptc antibody (*A*) or anti Shh antibody (*B*). The positions of Ptc and of the Shh-Ptc cross-linked complexes are indicated in the figure. Positions of molecular weight standards are indicated at the *left* of the panel.

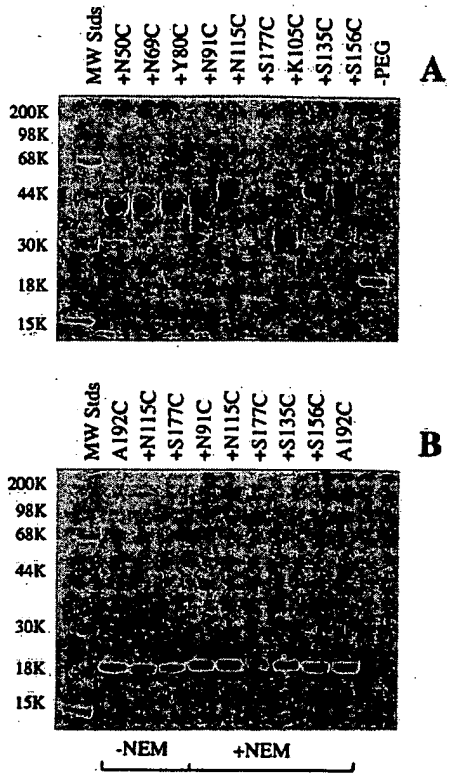


FIG. 4. **Characterization of the mAb 5E1-binding epitope on Shh.** The modified cysteine mutants of Shh were used to map the mAb 5E1-binding epitope. The modified proteins (*A*) after pegylation with 5000 PEG maleimide and (*B*) after treatment with *N*-ethylmaleimide were incubated with 5E1-Sepharose and the immune precipitates subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Lane designations correspond to the individual mutant analyzed. The −PEG designation in *B* is Shh C24II/A197C that had not been treated with PEG. *MW Stds* are prestained high molecular weight markers. Apparent masses of the molecular weight standards are indicated at the *left*.

293 cells (data not shown). Based on the sequence of the *ptc* construct, we expected Ptc to have an apparent mass of 170 kDa, consistent with the prominent band seen at that position. Whereas the other forms have been reported previously and presumably are related to Ptc (22, 30), their structures are not known. When the same blot was probed with anti-Shh antibody (Fig. 3*B*), all of the mutants except the parent protein C24II/A192C, N69C, and Y80C formed cross-linked complexes with Ptc as evidenced by the presence or absence of the band at 170–180 kDa. This band was not detected in cell lysates from Ptc-transfected or non-transfected 293 cells that had not been subjected to cross-linking (data not shown). The fact that most of the mutants formed a cross-linked complex with Ptc indicates that most of the surface of Shh is in relatively close proximity with Ptc. We have not characterized the other cross-linked bands that were detected by the anti-Shh antibody.

Although the results from cross-linking are similar to the blocking data we observed with the PEG-modified mutants, we had expected that the data would more closely resemble the effect of pyrene modification on activity. Several possibilities could account for the data. First, loss of function is a more rigorous test for association than cross-linking, since cross-linking only requires that the Shh and Ptc be localized within the span of the cross-linker, *i.e.* approximately 20 Å, during the lifetime of the activated cross-linker, whereas loss of function requires that the interaction be close enough that the probe can interfere with binding. Second, many of the probes used in these studies can assume a planar orientation because of free rotation around the bond between the nitrogen on the maleimide and the functional group. In this orientation the groups would be less disruptive than the size indicates. In addition to our analysis of sites on Shh that are associated with Ptc, the benzophenone-modified mutants should serve as valuable probes for mapping sites on Ptc that are associated with Shh as well as for studying interactions between Shh and other receptors such as Ptc-2 and HIP.

Characterization of the Binding Epitope of the Neutralizing Shh mAb 5E1—The pegylated Shh mutants were also used to map the binding epitope of mAb 5E1 (34) on Shh. In these studies, the pegylated mutants were incubated with 5E1-Sepharose (17) and the immunoprecipitates analyzed by SDS-PAGE for the presence of the mutants. All of the pegylated mutants except S177C were immunoprecipitated by the antibody, indicating that the 5E1 epitope is in the vicinity of Ser-177 (Fig. 4*A*). The epitope was further localized by subjecting the unmodified mutants and NEM-treated mutants to the same analysis (Fig. 4*B*). When the NEM-treated mutants were analyzed, again only the binding of the S177C mutant was affected. Surprisingly, even without modification of the S177C mutant there was a 2-fold reduction in the amount of the product that was immunoprecipitated, indicating that even the simple substitution of the serine hydroxyl group with a thiol was significant enough to effect 5E1 binding. Together these data indicate that Ser-177 forms part of the 5E1-binding site.

Structure-Activity Analysis of the Modification Data—Fig. 5 summarizes the structure-activity data for the modified forms of Shh with respect to the positions of the modified amino acids on the structure of Shh. Modification with 20,000 PEG (Fig. 5*A*) and with 5000 PEG (Fig. 5*B*) reveals that about a third of the surface, in particular the regions containing the C terminus, the $\beta1$–β' loop (mutant N69C), the β'–$\beta2$ loop (mutant Y80C), and the $\beta2$–β'' loop (mutant N91C) can be pegylated with no loss of function. Since a cholesterol moiety is attached at or near the C terminus in the membrane-tethered form of Shh, the lack of effect of modifications at these sites on function

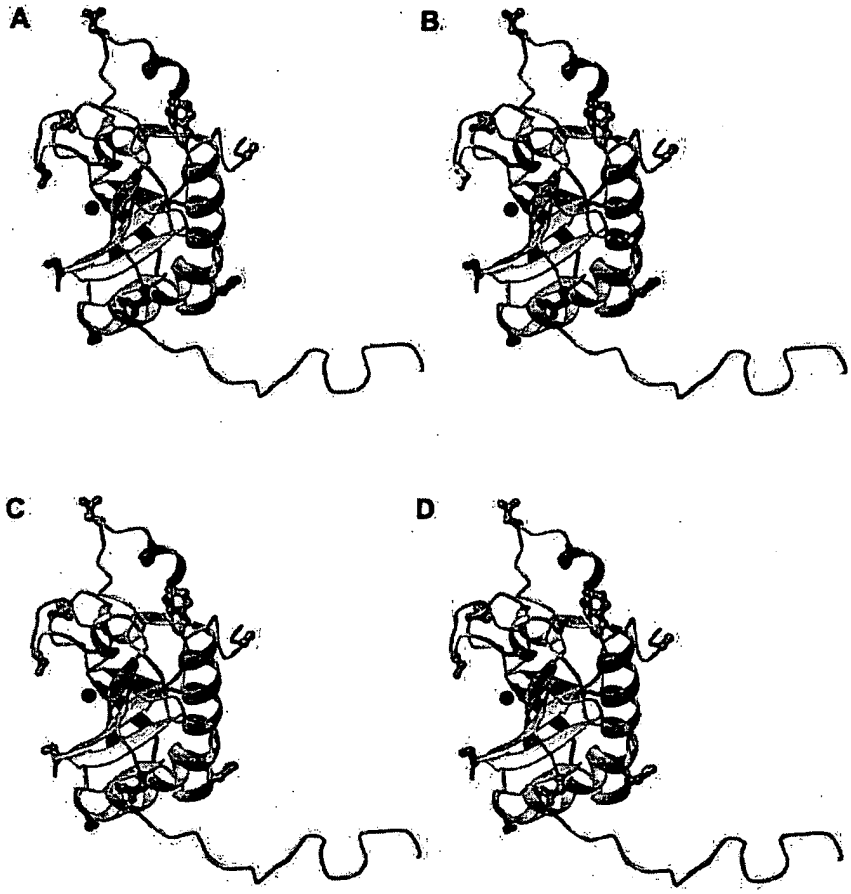


FIG. 5. **Structure-activity analysis of the modification data.** The structure-activity data for the 10 Shh cysteine mutants shown in Tables I and II that were used to study Ptc-Shh interactions are mapped on the model for the structure of Shh shown in Fig. 1. The positions of the mutated amino acids are indicated in *green* if the modification did not effect function, in *yellow* if the modification resulted in a partial inactivation, and in *red* if the modification inactivated the Shh. *A*, 20,000 PEG data. *B*, 5000 PEG data. *C*, pyrene data. *D*, mAb 5E1 binding data.

supports the hypothesis that this region on Shh interacts with the cell membrane. All other sites are sensitive to pegylation. Of the six sites where pegylation affected function, only two (N50C and S156C) lost activity following modification with pyrene maleimide (Fig. 5*C*). These sites are located on surface loops on either side of where the N terminus protrudes from the surface of the protein. We have shown previously that the N-terminal cysteine is a critical element for function, and modifications at this site can result in up to a 30-fold increase in potency (17) or loss of function (30) when evaluated in the C3H10T1/2 assay. The published crystal structure of murine Shh (20) is of an N- and C-terminally truncated form of the protein containing residues 34–195, and the first residue visible in the electron density was lysine 39. Thus, no information on the N terminus could be elucidated from the murine structure. In the crystal structure of the human Shh C24II mutant, the entire N terminus is visible in the electron density (Fig. 1).[3] A striking feature of the N-terminal region is its extended conformation; the N-terminal amino group is located approximately 30 Å away from the globular domain of Shh. This unusual conformation is stabilized by crystal contacts. Specifically, Phe-30 and Pro-26 make extensive hydrophobic contacts with an aromatic region containing residues Phe-47, Trp-172, Tyr-174, and His-182 near the metal binding cleft of a symmetry-related molecule.

Recently, Beachy and co-workers (35) published a study where they mapped Ptc-binding regions on Shh by conventional mutagenesis using evolutionarily conserved surface residues as targets. In their study, four mutants were generated that together contained 20 different mutations. Each mutant defined a geographic surface of Shh (referred to as SA, SB, SC, and SD). Of these, only the SC surface contributed significantly to Ptc binding. Three additional mutants were generated that each contained two of the six SC mutations. These additional mutants exhibited clear but reduced effects on Ptc binding and signaling. Here, using steric interference to map Ptc-Shh interactions, we find that the same overall region of Shh is involved in Ptc binding. Two of the sites that we mutagenized (Ser-156 and Ser-177) are on the SC surface, and modifications to both affected function. Ser-156 was particularly sensitive to modification as evident by the large reduction in potency following modification with pyrene maleimide. Modifications to the S177C mutant only affected function when the larger PEG attachments were used. In our analysis, modification of N50C with pyrene also interfered with Ptc-Shh interactions. Asn-50 is near Ser-156 in the Shh structure and lies on the interface between the SB and SC surfaces. Together with the data from Fuse *et al.* (35), our findings stress the importance of the region in Shh that is defined by Ser-156 and Asn-50 for Ptc binding. We also determined that N115C, which lies in the SA surface, and Gly-169 and Ser-135, which were outside of the regions tested, affected binding but only when modified with the larger PEG attachments. These findings, together with our data from cross-linking, suggest that Shh-Ptc interactions may cover a larger surface of Shh than was previously predicted.

In addition to mapping sites on Shh that interact with Ptc, we also used the cysteine mutants to characterize the binding epitope for mAb5E1, a neutralizing antibody that disrupts binding of Shh to Ptc. Whereas the 5E1 site was localized to within the SC surface by Fuse *et al.* (35), we determined that Ser-177 forms part of the binding epitope and thus further localized the binding site within this region. Our data reveal that 5E1 binding and Ptc binding are at distinct but close and potentially overlapping sites on Shh.

A significant advantage of the mapping analysis by steric interference over conventional mutagenesis studies is that the proximity of a specific group on a ligand with its receptor can be mapped even if the ligand residue and the receptor are not in direct contact. This is highlighted here by the fact all of the Shh cysteine substitution mutants tested were active prior to modification, but with each incremental increase in the size of the probe, modification blocked function in a larger percentage of the mutants. Since the pattern of inhibition seen with each probe is a subset of that seen with the next larger size probe, the changes in the patterns presumably reflect differences in the distance between the interacting sites. This notion is particularly apparent for the smaller probes that were tested. Although we successfully utilized the 5000 and 20,000 PEG maleimide agents to map Shh-Ptc interactions on a gross level, the linear structure and flexibility of the PEGs limit their utility for this type of analysis. One approach that would greatly enhance the method would be to rationally design a series of small globular probes of rigid structure that could be used to define more precisely actual distances between interacting sites.

Although the concept of mapping by steric interference stems from antibody strategies for epitope mapping where antibodies frequently block function through steric effects rather than by directly binding at active sites, this is the first study to our knowledge where targeted probes of varying sizes have been used in such a manner. Whereas conventional mutagenesis (35) and the steric interference strategy described here both allow for the mapping of functional epitopes within the Shh structure, loss of function by mutagenesis is often difficult or impossible to attain if a large surface of a protein is involved in binding, and in several instances, mutagenesis studies have produced erroneous conclusions because loss of function was unrelated to ligand/receptor binding (37). The steric interference method we describe should provide an alternative to these types of approaches.

In summary, we have used a novel method probing structure-function relationships for Shh-Ptc interactions where we mutagenized Shh and inserted cysteines at surface positions that could then be specifically derivatized with groups of different sizes and shapes. The effects of the modifications on function were then determined. Based on these analyses, key regions in the structure that were involved in Ptc binding and 5E1 binding were mapped that give a new view of how Shh interacts with Ptc. The mapping strategy should be readily applicable to any protein but is likely to prove particularly valuable where structural data exists.

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(54) TOPICAL TREATMENT OF PSORIASIS USING NEUTRALIZING ANTIBODIES TO IL-8

LOKALE BEHANDLUNG VON PSORIASIS UNTER VERWENDUNG NEUTRALISIERENDER ANTIKÖRPER GEGEN IL-8

TRAITEMENT TOPIQUE DU PSORIASIS METTANT EN OEUVRE DES ANTICORPS APTES A NEUTRALISER IL-8

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Exhibit 3

Description

FIELD OF THE INVENTION

[0001] The invention relates to pharmaceutical compositions for topical application of mAb to treat psoriasis and other inflammatory skin conditions.

BACKGROUND OF THE INVENTION

[0002] Psoriasis is a common, noncontagious, chronic inflammatory disease of unknown cause. It is a worldwide disease and it's prevalence in the general population is nearly 3% for the people of the Faroe Islands and Denmark. Over 5 million people in the United States are afflicted with this disease (2% of the population).

[0003] It most commonly appears as sharply circumscribed salmon pink patches covered with silvery white scales. Diagnosis is usually made by observation and examination of the skin. There are different types of psoriasis that display certain characteristics. Each of these types can range from mild to severe. However, psoriasis is variable and one type can change into another type or several types can exist at the same time. The National Psoriasis Foundation describes the types as follows:

(1) *Plaque Psoriasis:* raised, inflamed lesions that are covered in white scale. Most common types that is also called psoriasis vulgaris. Locations: anywhere, but usually on scalp, elbows, knees, trunk.
(2) Guttate Psoriasis: small, drop-like dots with some scale. Location: trunk, legs, arms.
(3) Inverse Psoriasis: smooth inflamed lesions, no scale. Location: skin folds, armpit, groin.
(4) Erthrodermic Psoriasis: severe sloughing of the skin with redness. Location: anywhere on body.
(5) Psoriatic Arthritis: swelling and inflammation of joints can result in 10% of psoriasis patients. Location: knees, hips, elbows, spine, hands and feet.
(6) Scalp Psoriasis: is usually plaque type. Affect 50% of psoriasis patients.
(7) Nail Psoriasis: pitting, discolouration, and loss of fingernails and toenails. Usually inflammation of skin around the nail.

[0004] It has been reported that a combination of genetic, environmental, and immunological factors contribute to the disease. It is believed that a person is predisposed to developing psoriasis, but there appears to be no pattern of inheritance. Only one in three people reports a family history of this disease, whereas others show no incidence of psoriasis in family. There may be important triggers (superantigens such as bacteria, virus, and fungus; vaccinations, intramuscular injection, certain drugs, stress, and injury to the skin; Koebner phenomenon, etc.) that initiate the development of psoriasis in those that are predisposed to developing it. Then as a result of these triggers the immune system causes excessive skin cell reproduction.

[0005] In normal skin growth, skin cells produced in the basal cell layer move up through the epidermis to the outermost layer, the stratum comeum. This process from cell birth to cell death takes about 28-30 days. When skin is damaged this cycle is much faster. Although there is no wound at the site of psoriatic lesions, skin cells called keratinocytes act in a regenerative manner. New skin cells are produced in 2-4 days, thus making it very difficult shed old cells at a adequate rate. The elevated scaly lesions are a result of the buildup of cells. The white scale is dead skin cells and the redness is a result of an increase in blood flow to areas of high cell division.

[0006] Psoriasis is characterized by (1) extreme epidermal hyperproliferation (excessive growth associated with incomplete and accelerated differentiation) (2) noticeable inflammation of epidermis and dermis at local sites with development of neutrophil microabscess and enhanced induction of cycling T lymphocytes. Thus, the cause of psoriasis was initially thought to involve one of the mediators of hyperproliferation However research began to focus on the immune system after by chance it was discovered that cyclosporine immunosuppressive effects significantly improved psoriasis in patients. Thus it is now viewed as an autoimmune disease. Recently, there has been more elucidation about the pathogenesis of psoriasis. Today there are three main theories of psoriasis origin and development. (1) T-lymphocytes are activated in psoriatic lesions by cytokines that are released from epidermal keratinocytes. (2) Antigen dependent T-cell activation causes the release of cytokines that activate epidermal keratinocytes. (3) Autoimmune reactions of CD8+ "killer" T-lymphocytes with epidermal keratinocytes trigger epidermal activation.

[0007] Psoriasis does not affect overall health and is not life threatening, but people do die from complications associated with this disease. The physical and especially the emotional effects of psoriasis can be painful. This disease can cause disfigurements which physically limit, thus affecting job and leisure activities. This causes frustration, embarrassment, fear and depression for psoriasis sufferers, especially with severe types. Psoriasis is persistent and unpredictable in its course, thus no single treatment works for everyone. As a result, there are a variety of treatments available that can be used alone or in combination. These treatments may diminish symptoms transiently but they are not curative. Very often they are aesthetically unpleasant, expensive, time consuming and have side effects that are unhealthy. For the most part, present treatment is unsatisfactory.

[0008] In general, mild forms of psoriasis are treated by topical applications of glucocorticoids eg. Corticaine. Keratolytic agents such as sulfur or salicylic acid, are useful adjuvants. Side-effects are mild. Moderate forms of psoriasis are usually treated with anthralin/dithranol or tar preparations eg. Pentrax. Side-effects are mild-

moderate. Severe cases of psoriasis or mild to moderate forms that do not respond to conventional therapy may require treatment with systemic medication. Side-effects are usually severe.

[0009] Some of the current therapies of psoriasis are as follows:

1. Phototherapy:

(a) Narrow ban ultraviolet B phototherapy (UVB): burning and carcinogenesis.
(b) Psoralen with ultraviolet A (PUVA): long term problem of carcinogenesis and short term problems of nausea, phototoxicity, and pruritus
(c) Photodynamic therapy: limitations include photosensitivity and tissue destruction.

2. Drugs approved for other uses:

(a) Zidovudine (Retrovir): used to slow AIDS. Side effects involved a decrease in RBC and WBC counts.
(b) Histamine$_2$ Receptor Antagonists: used to treat stomach ulcers, i.e. ranitidine (Zantac) and cimetidine (Tagament). Side effects involved an initially worsening of symptoms.
(c) Antithyroid Thioureylenes: used for hyperthryroidism, i.e. propylthiouracil and methimazole (Tapazole). Side-effects: hypothyroidism, but decreased with a topical formulation
(d) Capsaicin (Zostrix 0.025% cream): is approved for pain relief in rheumatoid arthritis, osteoarthritis, and neuralgia. The major side effect is stinging.

3. New drugs developed for psoriasis:

(a) Acitretin (Neotegison/Neotigason): a second-generation monoaromatic retinoid. This drug is teratogenic. Related retinoid Etretinate (Tegison/ Tigason) shows similar effects.
(b) Fumaric acid therapy: side effects include abdominal disturbances, lymphopenia, flushing, and mild change of hepatic and renal function. In 85% of patients, long term therapy causes lymphopenia.
(c) Vitamin D derivatives: 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) shows hypercalciuria in systemic and topical applications. A synthetic 1,24-dihydroxyvitamin D_3 analogue, i.e. Calcipotriene ointment (Dovonex ointment) diminishes hypercalciuria side-effects but results in face and intertriginous irritation. Tacalcitol, also shows face irritation.
(d) Tazarotene (Tazorac): is an acetylenic retinoid molecule. Topical application showed dose-related irritation.

4. Immune therapy:

(a)Cyclosporine (Sandimmune): is approved for use in organ transplantation. Some side effects are potentially toxic and are as follows: headaches, gastrointestinal disturbances, hypertrichosis, paresthesias, and gingival hyperplasia. It is extremely important that nephrotoxicity be carefully monitored with this drug. Side-effects increase with length of time the drug is administered, so it is not an acceptable long-term therapy for patients. A new formulation called Neoral (approved for organ transplantation) may reduce toxicity, but further studies are needed.
(b)DAB_{389}IL-2: a cytotoxin that selectively attacks IL-2 receptors on cells and destroys them. Side effects include: flu-like symptoms, pruritus, and transient transaminase elevation.
(c)Tacrolimus (Prolaf): is a macrolide antibiotic used to treat allograft rejection in liver transplant patients. Side-effects are similar to Cyclosporine.
(d)CTLA41g: is an experimental agent that blocks the second signal in T-cell activation. Side-effects are unknown. Clinical trials are in progress.
(e)Anti-CD4 Monoclonal Antibody: side effects include chills and fever. More in depth toxicity studies are needed.
(f)T-cell receptor peptide vaccines: Vβ3 and Vβ13.1 T-cells are targeted. Clinical trials in progress to determine toxicity
(g)Other immunologic agents: include TNF-alpha inhibitors and antisense oligonucleotides. Side-effects unknown.

[0010] Monoclonal antibody (mAb) preparations may be effective in fighting malignancy, infection, and immune disorders. A monoclonal antibody is directed against and binds to a single epitope on an antigenic molecule. Characteristics such as homogeneous high binding affinity and specificity make them suitable for developing therapeutics. However, mAb preparations for the most part have been administered using systemic drug delivery methods.

[0011] Topical treatments are preferred for treating psoriasis and other skin diseases because there are less side-effects. A concerted effort to develop a topical preparation containing antibodies for treating psoriasis has not been undertaken. This is because it has been accepted that a sufficient level of antibodies cannot be absorbed through the skin to combat psoriasis.

[0012] It is unknown exactly which biological factors play a role in the manifestation of the disease. This has made it difficult to develop a topical treatment. With a topical treatment, lower levels of antibodies reach the target site. A topical treatment therefore requires the use

of an antibody or other active ingredients which can neutralize a biological factor which is directly linked to the manifestation of the disease.

[0013] We have found that interleukin-8 (IL-8) or neutrophil-activating protein (NAP-1) plays a significant role in the manifestation of psoriasis and other inflammatory skin conditions. It was not previously known that antibodies or other agents that neutralize IL-8 are effective in the treatment of psoriasis and other inflammatory skin conditions.

[0014] There is therefore a need for a topical treatment for treating psoriasis that is effective in neutralizing biological factors that are directly involved in the manifestation of the disease. There is a specific need for a topical treatment for psoriasis that contains antibodies for neutralizing IL-8.

SUMMARY OF THE INVENTION

[0015] The present invention provides a pharmaceutical composition for treating a human subject for psoriasis or other inflammatory skin conditions. The composition comprises an agent that diminishes the effect of psoriasis or other inflammatory skin conditions together with a pharmaceutically acceptable carrier. The present invention provides a method of treating psoriasis or other inflammatory skin conditions through topical administration of a pharmaceutical composition that diminishes the effect of these conditions.

[0016] According to one aspect of the present invention, a pharmaceutical composition for topical administration to a patient to treat an inflammatory skin condition is provided. The composition comprises an antibody that diminishes the effect of the inflammatory skin condition. The composition also includes a pharmaceutically acceptable carrier.

[0017] According to another aspect of the present invention, a pharmaceutical composition for topical administration to a patient to treat psoriasis is provided. The pharmaceutical composition comprises an antibody that neutralizes interleukin-8.

[0018] According to another aspect of the present invention, a pharmaceutical composition for topical administration to a patient to treat psoriasis is provided. The pharmaceutical composition comprises at least one of the following antibodies:

- I8-60
- I8-S2
- 3C6

and a pharmaceutically acceptable carrier.

[0019] According to another aspect of the present invention a method of treating psoriasis or other inflammatory skin conditions is provided. The method comprises the step of applying topically a pharmaceutical composition comprising an antibody that neutralizes interleukin-8 and a pharmaceutically acceptable carrier.

[0020] According to yet another aspect of the present invention a method of treating psoriasis is provided. The method comprises the step of applying topically a pharmaceutical composition comprising an antibody is effective in diminishing the effects of psoriasis and a pharmaceutically acceptable carrier.

[0021] According to yet another aspect of the present invention, use is made of a pharmaceutical composition comprising an antibody that neutralizes interleukin-8 for topically treating psoriasis or other inflammatory skin conditions.

DESCRIPTION OF THE FIGURES

[0022]

Figure 1 is a table showing the isotypes of the IL-8 monoclonal antibodies that were identified with Mouse typer sub-isotyping kit;

Figure 2 is a table indicating that monoclonal antibodies 18-S2, 18-60, and 3C6 recognize different epitopes of IL-8 molecule;

Figure 3 is a table setting out the reagents used to prepare a base cream;

Figure 4 is a table summarizing effects of a topical composition containing monoclonal antibodies on psoriasis patients;

Figure 5 is a table summarizing effects of a topical composition containing polyclonal antibodies on psoriasis patients; and

Figure 6 is a table summarizing effects of a topical composition containing polyclonal antibodies on eczema patients.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The monoclonal antibodies outlined in this invention are obtained according to processes that are known per se. General hybridoma techniques are well known, however in certain cases specific problems may require changes to known techniques. There is no certainty that the required hybridoma will be formed and produce specific antibodies, but the degree of success will depend on the completion of the following steps:

(a) Mice were immunized with purified recombinant human interleukin-8 (IL-8, monocyte-derived, 72 a. a. form). The immunization schedule and the IL-8 concentration ("immunogen") should be sufficient to produce satisfactory serum titers of antibodies. Three immunizations with approx. 200 µL of antigen solution every 3-4 weeks by subcutaneous (s.c) and intraperitoneal (i.p.) injection have been found to be effective.

(b) Using well known experimental techniques the spleen cells of the immunized mice were removed 3-4 days after last ("booster") immunization and suspended in an appropriate medium.

(c) The suspended spleen cells are fused with mouse myeloma cells of a suitable cell line with a suitable fusion promoter, preferably polyethylene glycol (PEG) having a molecular weight from 1000 to 4000 . However, other fusion promoters known in the art may be used. Preferably, spleen cells are fused with myeloma cells in a 5:1 ratio.

Any appropriate mouse myeloma cell line may be used but it is preferred that myeloma cells that do not survive in a selective culture medium containing hypoxanthine, aminopterin and thymidine (HAT) medium be used, such as those that lack enzyme hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) or the enzyme thymidine kinase (TK). Especially preferred are myeloma cells and cell lines that do not survive in HAT medium and do not by itself secrete any antibody, for example the cell lines X63-Ag8.653 and Sp2/0-Ag14.

After fusion, the cells were cultured in selective HAT medium, which supports the growth of hybridoma cells, not the growth of unfused myeloma cells. Only fused cells continue to grow because they have from the myeloma cells the ability to grow in vitro, and from the spleen cells parent the ability to survive in selective medium.

Hybridoma cells must be grown in suitable culture media, for example RPMI 1640 medium or Dulbecco's Modified Eagle's Medium. This media is supplemented with 10-15% fetal bovine serum. At the beginning of cell growth "feeder cells" may be added, for example spleen cells, bone marrow, normal mouse peritoneal exudate cells or "hybridoma growth factors".

(d) As soon as the medium has started to turn acidic (yellow) and the cell colonies are visible, a small amount of the cell culture supernatant should be removed to be tested for the presence of the desired antibodies, for example antibody to IL-8.

(e) Wells that test positive for antibody through the screening assay are selected and cloned as soon as possible using well known experimental techniques, for example limiting dilution (easiest) in order to ensure their monoclonality.

(f) The mAb's isotypes were determined using well known methods, for example "dipstick" assay/dot blot or ELISA.

(g) Antibodies were tested for their ability to specifically neutralize human IL-8 activity. Three hybridoma cell lines produced murine antibodies with high neutralizing ability and were deposited at the ATCC, under the Budapest Treaty Deposit Procedure, on May 14 1998, under accession numbers CRL-12528, CRL-12527 AND CRL-12529 for the cell lines designated 18-60 (IL-8-60), 18-S2 (IL-8-S2), and 3C6 (IL-8-3C6), respectively.

(h) 18-60, 18-S2, and 3C6 which bind to different antigenic determinants of IL-8 can be utilized in a "cocktail" for immunotherapy of psoriasis and other inflammatory skin conditions. It is suggested to administer topically to patients suffering from psoriasis and other inflammatory skin conditions, a combination of said antibody together with a pharmaceutically acceptable carrier. Preliminary clinical trials have demonstrated that such a topical composition is effective.

(i) To ensure a good stock of hybridoma cells and the antibody it secretes, it is necessary to grow up the cells after repeated clonings. This can be done by production of ascites fluid or bulk tissue culture. For ascites production the preferred hybridoma is injected into mice, which will grow and cause ascitic fluid containing mAb to form in the abdominal cavity. After a suitable length of time mouse ascites can be collected using well known experimental techniques and may provide up to 10 mg/mL of antibody. For bulk culture, the hybridoma clones are cultured in vitro in a suitable medium using static cultures, roller cultures or bioreactors. After a suitable length of time the supernatant from a standard flask can be collected and may provide between 10 to 50 µg/mL.

(j) The bulk antibodies should be purified using well known experimental techniques to remove all major contaminants, for example affinity chromotography.

(k) A polyclonal antibody may also be used quite satisfactorily as an alternative to the monoclonal antibody "cocktail" for immunotherapy of psoriasis and other inflammatory skin conditions. Polyclonal antibody was prepared by injection of chicken with purified recombinant human interleukin-8 using standard immunization protocols. After a suitable period of time eggs were collected and the chicken yolk IgY was purified. It is suggested to administer topically to patients suffering from psoriasis and other inflammatory skin conditions, a combination of said polyclonal antibody together with a pharmaceutically acceptable carrier. Preliminary clinical trials have demonstrated that such a topical composition is effective.

[0024] The following examples illustrate the preferred embodiments of the invention without limiting the scope thereof.

EXAMPLE 1

PRODUCTION OF MOUSE ANTI-HUMAN IL-8 MONOCLONAL ANTIBODIES

1.1 Immunogen:

[0025] Purified recombinant human interleukin-8 (rh-IL-8) derived originally from human monocyte was obtained from Pepro Tech, USA. It consists of 72 amino acids, has a molecular weight of 8.5 kDa, purity > 98% by N-terminal assay and SDS-PAGE silver staining,

showed strong chemotactic activity to human neutrophils by chemotaxis assay. 1.2 Immunization:

[0026] Female BALB/c mice (Charles River Laboratories, Inc., Canada) were immunized with rhIL-8. The immunization procedure was as follows: 200 μL of antigen (20μg rhIL-8/200μL PBS) added to 200μL Freund's Complete Adjuvant to make 400μL antigen emulsified solution. Day 1: this solution was injected subcutaneously (s.c.) into mice at multiple sites on back. Day 27: 400μL antigen solution (20 μg rhIL-8/400μL PBS) was added with 400μL Freund's Incomplete Adjuvant, and mice immunized by intraperitoneal injection (i.p.). Day 59: same as day 27. Day 91: same as day 27. Day 152: immunize mice by i.p. injection with 20 μg rhIL-8/450 μL PBS antigen solution. Day 155: the spleens of the mice were removed and prepared for cell fusion.

1.3 Cell fusion:

[0027] Mouse SP2/0-Ag 14 myeloma cells (ATCC, CRL 1581) which don't secret heavy or light chain of immunoglobulins were used. It is resistant to 8-azaguanine and fails to survive in HAT medium. SP2/0-Ag14 is widely used as fusion partner to prepare mAb secreting hybridoma.

[0028] SP2/0-Ag14 myeloma cells in logarithmic phase were washed with serum-free RPMI 1640 medium twice.

[0029] Spleen cell suspension prepared under sterile conditions were washed with serum-free RPMI 1640 medium twice.

[0030] Spleen cells and SP2/0-Ag14 cells were mixed in a 5:1 ratio, then centrifuged at 1500 RPM for 7 minutes and supernatant removed. Slowly, 1 mL of 50% PEG4000 (MW:3000-4000) was added (GIBCO BRL, USA), taking 1 minute. The mixture was let to sit for 1.5 min. 5 mL of serum free RPMI 1640 medium was added slowly, taking 2.5 min. The mixture was let to sit for 5 min. The mixture was centrifuged at 1000 RPM for 5 minutes and supernatant removed. Cells re-suspended in regular RPMI 1640 medium containing 15% FBS (GIBCO BRL).

[0031] The above cell suspension was distributed 100 μL (2 drops/well) to the 96 well plates (100 μL, well). Plates were placed in a CO_2 incubator (37°C), then HAT culture media was exchanged every 3 days. At day 10, HAT culture media was exchanged for HT media.

[0032] After 14 days of incubation, supernatant from wells with growing colonies are screened for their binding capacity to IL-8 with ELISA and anti-IL-8 positive clones were selected.

1.4 Cloning of hybridoma:

[0033] In limited dilution method, the diluted cell suspension (3-10 cells/mL) was added 2 drops/well to 96 well plate. The plate was then incubated in a CO_2 incubator (37°C). During incubation, every well was exchanged with 1/3 fresh culture RPMI 1640 culture media every 3-4 days. Ten days later, the second screen and cloning were carried out. Clones whose mean cloning rate is <66.7 and mean antibody positive rate is 100% were deemed monoclonals after three successive clonings. There were 9 clones that were deemed monoclonals specific for human IL-8,

EXAMPLE 2

CHARACTERIZATION OF MONOCLONAL ANTIBODIES

2.1 Immunoglobulin subtypes:

[0034] The isotypes of the IL-8 mAbs were identified with the Mouse typer sub-isotyping kit (BioRad, USA). The results are summarized in Figure 1.

2.2 Specificity:

[0035] All IL-8 mAbs were tested for cross-reaction to various cytokines and chemotactic factors by ELISA. Results showed that those IL-8 mAbs exhibited no cross-reaction with IL-1β, IL-7, IL-16, EGF, M-CSF, GM-CSF, MCAF, MCP-3, TGF-β1, TNF-α and BSA and were specifically reactive to IL-8.

EXAMPLE 3

MONOCLONAL ANTIBODY NEUTRALIZING TESTS

[0036] The following anti-IL-8 mAbs showed a strong neutralization effect on IL-8:

(a) 18-60 and 3C6: purified antibodies were used in a neutrophil chemotaxis assay. rhIL-8 at 1 μg/mL was incubated with different concentrations of purified 18-60 or 3C6 at 37 deg.C for 45 minutes, then diluted to a final concentration of 50 ng/mL, an optimal dose of rhIL-8 for eliciting neutrophil responses. 26 μg/mL of monoclonal antibodies neutralized 50% of neutrophil chemotactic response to rh IL-8 with 100% neutralization at 80 μg/mL.

(b) 18-S2: through flow cytometry analysis it demonstrated IL-8 could activate human granulocytes and induce elevation of $[Ca^{2+}]$. Neutralizing effect can thus be determined by blocking $(Ca^{2+})_i$ elevation by anti-IL-8 antibodies. Tests for changes of Ca++ concentration in cells with flow cytometry showed that 18-S2 mAb displayed a high neutralizing effect on human granulocytes activated by IL-8.

EXAMPLE 4

EPITOPE RECOGNITION OF MONOCLONAL ANTIBODIES WITH NEUTRALIZING ACTIVITY

[0037] Recombinant IL-8 at a concentration of 0.02 µg/mL were coated on a 96 well microplate. Using ELISA Additivity Test [Friguet, B. et al. (1983) J. Immunol. Methods, 60:351-358.] index (AI)=[(2OD_{1+2})/(OD_1 + OD_2) -1] x 100% was determined. Using this method, an AI>50% indicates that the two antibody clones recognize different epitopes. Results from Figure 2 indicate that mAbs 18-S2, 18-60, and 3C6 recognize different epitopes of IL-8 molecule.

EXAMPLE 5

BULK PRODUCTION & PURIFICATION MONOCLONAL ANTIBODIES WITH NEUTRALIZING ACTIVITY

[0038] To produce larger quantities of mAbs 18-60, 18-S2, 3C6 the hybridomas were grown in mice.
Day 1: Balb/c mice were injected intraperitoneally with 0.5 mL of Pristane (2,6,10,14-tetramethylpentadecane).
Day 7: the hybridoma cells were washed with PBS and 1 x 10^6 cells were injected into each mouse using i.p. route. After two weeks the ascites fluid was removed using well known experimental techniques.

[0039] Recombinant Protein G Agarose (GIBCO BRL, USA) was used to purify IgG antibody from cell culture supernatant or ascites. Binding Buffer (Sodium Phosphate, pH 7.0/0.15M Sodium Chloride) and Eluting Buffer (0.1M Glycine Hydrochloride, pH 2.6) were used for purification.

[0040] For every batch of mAbs purified by protein G affinity chromatography the specificity and binding affinity were tested by ELISA method. IL-8 was coated at 0.1 µg/well and the mAb titers at their end point were found to be 0.1 ng/mL (18-60), 0.1 ng - 1 ng/mL (18-S2), and 100 ng/mL (3C6). Purified and quality controlled mAbs were filtered by 0.22µm Sterile Millex-GS filter for sterilization (Millipore, Canada), then lyophilized and stored at -20°C.

EXAMPLE 6

PRODUCTION OF CHICKEN ANTI-HUMAN IL-8 POLYCLONAL ANTIBODIES

6.1 Immunogen:

[0041] Purified recombinant human IL-8.

6.2 Immunization:

[0042] Shaver Browns laying hen was immunized with recombinant human IL-8. The immunization procedure was as follows: 200 µg IL-8 dissolved in PBS was emulsified with an equal volume of Complete Freund's Adjuvant and a total of 600 µL was injected subcutaneously (s.c) and intramuscularly (i.m.) in various combinations in 4 sites on each breast. Day 17, 29, 42, 56, 118: the hen was boosted with 100 µg of IL-8 emulsified with an equal volume of Freund's Incomplete Adjuvant Day 187: the hen was boosted with 200 µg of IL-8 emulsified with an equal volume of Freund's Incomplete Adjuvant. The hen eggs were collected and the egg yolk IgY was purified by Gallus Immunotech, Canada.
6.3 Specificity: The polyclonal antibody exhibits no detectable cross-reactivity with human serum albumin, and other cytokines tested. .
6.4 Neutralizing Activity: The purified IgY from egg yolks showed potent activity of neutralizing IL-8. Total chicken IgY fraction at 50 µg/mL showed 60% inhibition of 10 ng/mL IL-8 induced IL-8 RB/293 cell migration.
6.5 Product Form: Chicken IgY in phosphate buffered saline, pH 7.3 no preservatives. Stored at -20°C.

EXAMPLE 7

PHARMACEUTICAL PREPARATION FOR TOPICAL ADMINISTRATION

7.1 Preparation of base cream:

[0043] The reagents from figure 3 were weighed and placed in an open stainless steel tank (2000 mL vol.) successively.

[0044] The stainless steel tank was placed into a thermostat water bath and heated to 80°C which took approximately 10 minutes. The liquid is thoroughly mixed then emulsifying and homongenating equipment was placed into the open stainless steel tank, the mixture was stirred for 20 minutes at 3500 rpm until fully emulsified. The temperature of the thermostat water bath was cooled naturally to 30-37°C, until the mixture became a semi-solid cream. The mixture is being continually stirred.

7.2 Preparation of liquid antibody mixture no.1:

[0045] MAbs I8-S2, 3C6, and I8-60 are prepared in accordance with Example 5. For 1000 gm of base cream, 45 mg of total antibody was required, for example 15 mg (clone 18-S2), 15 mg (clone 3C6), and 15 mg (clone 18-60).

[0046] Add 4 mL of distilled water to antibody mixture per 100 gm of base prepared, for example 40 mL of distilled water was added to 45 mg antibody mixture to reconstitute to a final concentration of 1.125 mg/mL.

7.3 Preparation of liquid antibody mixture no.2:

[0047] Polyclonal Chicken Anti-human IL-8 is prepared in accordance with Example 6.. For 1000 gm of

base cream, 450 mg of polyclonal antibody was required. A higher mg/gm of cream was needed for polyclonal preparation because about less than 10% specific antibody to IL-8 was contained in whole IgY.

[0048] Add 4 mL of distilled water to antibody mixture per 100 gm of base prepared, for example 40 mL of distilled water was added to 450 mg antibody mixture to reconstitute to a final concentration of 11.25 mg/mL.

7.4 Preparation of topical composition (monoclonal)

[0049] While the base cream is being stirred using emulsifying and homongenating equipment, the liquid antibody mixture no.1 prepared in accordance with Example 7.2 is dropped to base cream prepared in accordance with Example 7.1 using a pasteur aspirating tube. After the antibody mixtureis added, the total mixture is stirred for 10 more minutes. The topical composition is packaged and stored at 4°C.

7.5 Preparation of topical composition (polyclonal)

[0050] While the base cream is being stirred using emulsifying and homongenating equipment, the liquid polyclonal antibody mixture no.2 is prepared in accordance with Example 7.3 is dropped to base cream prepared in accordance with Example 7.1 using a pasteur aspirating tube. After antibody mixture is added, the total mixture is stirred for 10 more minutes. The topical composition is packaged and stored at 4°C.

EXAMPLE 8

TREATMENT OF HUMAN PSORIATIC SKIN USING TOPICAL COMPOSITION (MONOCLONAL)

8.1 Materials and Clinical Protocol:

[0051] The topical composition was prepared in accordance with Example 7.4. The composition was applied to 29 psoriasis patients (23 plaque, 4 erythrodermic, and 2 arthritic). All patients received approximately 0.2g cream/cm^2 of lesion area. The cream was applied twice a day for 4 weeks.

[0052] Evaluation of effects were divided in 4 grades. These are cured, obvious effective, effective, non-effect.

cured: plaque diminished completely, pruritus disappeared
obvious effect: ≥ 60% plaque diminished, pruritus slightened (softened)
effective: 20% ~ 60% plaque diminished, pruritus slightened (softened).
non-effect less than 20% plaque diminished or exacerbation of psoriasis. Pruritus not softened or deteriorated.

8.2 Results of trials:

[0053] A summary of the effects of the topical composition on the 29 psoriasis patients are shown in figure 4.

[0054] The topical composition showed an obvious effect for erythrodermic psoriasis and arthritic psoriasis and may be effective for plaque psoriasis to some degree. No visible side-effects were observed.

[0055] This method of treating psoriasis is external, convenient and easy to administer, and shows effectiveness in a short period of time.

EXAMPLE 9

TREATMENT OF HUMAN PSORIATIC SKIN USING TOPICAL COMPOSITION (POLYCLONAL)

9.1 Materials and Clinical Protocol:

[0056] The topical composition was prepared in accordance with Example 7.5. The composition was applied to 8 psoriasis patients (4 plaque, 2 erythrodermic, and 2 arthritic) All patients received approximately 0.2 g cream/cm^2 of lesion area. The cream was applied twice a day for 4 weeks.

[0057] Evaluation of effects were divided in 4 grades. These are cured, obvious effective, effective, non-effect.

cured: plaque diminished completely, pruritus disappeared
obvious effect ≥ 60% plaque diminished, pruritus slightened (softened)
effective: 20% - 60% plaque diminished, pruritus slightened (softened).
non-effect less than 20% plaque diminished or exacerbation of psoriasis. Pruritus not softened or deteriorated.

9.2 Results of trials:

[0058] A summary of the effects of the topical composition on the 8 psoriasis patients are shown in figure 5.

[0059] The topical composition showed an obvious effect for erythromermic psoriasis and arthritic psoriasis and may be effective for plaque psoriasis to some degree. No visible side-effects were observed.

[0060] This method of treating psoriasis is external, convenient and easy to administer, and shows effectiveness in a short period of time

EXAMPLE 10

TREATMENT OF HUMAN ECZEMIC SKIN USING TOPICAL COMPOSITION (POLYCLONAL)

10.1 Materials and Clinical Protocol:

[0061] The topical composition was prepared in ac-

cordance with Example 7.5. The composition was applied to 8 eczema patients. All patients received approximately 0.2 g cream/cm^2 on lesion area. The cream was applied twice a day for 4 weeks.

[0062] Evaluation of effects were divided in 4 grade. These are cured, obvious effective, effective, non-effect.

cured: plaque diminished completely, pruritus disappeared
obvious effect: ≥ 60% plaque diminished, pruritus slightened (softened)
effective: 20% ~ 60% plaque diminished, pruritus slightened (softened).
non-effect: less than 20% plaque diminished or exacerbation of psoriasis. Pruritus not softened or deteriorated.

10.2 Results of trials:

[0063] A summary of the effects of the topical composition on the 8 eczema patients are shown in figure 6.

[0064] The topical composition showed an obvious effect in 63% of eczema patients. No visible side-effects were observed.

[0065] This method of treating eczema is external, convenient, and easy to administer. It shows effectiveness in a short period of time.

[0066] The mechanism by which anti-IL-8 antibody is absorbed into psoriatic lesions in a sufficient amount to neutralize IL-8 and be therapeutically effective is unclear. Possibly reasons include:

(1) high enough concentrations of antibodies can be maintained at the epidermal surface for a long period, thus permitting slow penetration of therapeutically effective dose.
(2) the defective permeability barrier in psoriasis may allow for greater penetration of an effective dose of antibodies into the epidermis.

[0067] The advantages of the proposed treatment over the present treatment are as follows:

(1) Topical application would minimize the toxic side effects that are often associated with systemic drug delivery because the treatment is applied locally.
(2) Antibodies are unique in that they are specific, homogeneous, and can be produced in vitro at infinitum.
(3) Antibodies to IL-8 specifically neutralizes IL-8 in psoriatic lesions, not anything else.

[0068] To produce a more effective cream, other neutralizing agents may be introduced into the topical composition. The neutralizing agent may be a IL-8 receptor blocking agent, for example a peptide that binds to IL-8 receptor site or antibodies to IL-8 receptor (IL-8R) or soluble IL-8 receptors.

[0069] Although the invention has been described with preferred embodiments, it is to be understood that modifications may be resorted to as will be apparent to those skilled in the art. Such modifications and variations are to be considered within the purview and scope of the present invention.

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Claims

1. A pharmaceutical composition for topical administration to treat an inflammatory skin condition, comprising an antibody that neutralizes interleukin-8.

2. A composition according to claim 1, wherein the antibody is a monoclonal antibody.

3. A composition according to claim 1, wherein the antibody is a polyclonal antibody.

4. A composition according to claim 1, wherein the antibody is an antibody fragment.

5. A composition according to claim 1, comprising at least one of antibodies 18-60 (ATCC accession No. CRL-12528), 18-S2 (ATCC accession No. CRL-12527) and 3C6 (ATCC accession No. CRL-12529).

6. A composition according to any preceding claim, which comprises a pharmaceutically acceptable carrier selected from a neutral sterile cream, gel, jelly, ointment, aerosol, patch and powder.

7. A composition according to any preceding claim, wherein the acceptable carrier is a cream.

8. A composition according to any preceding claim, wherein the condition is psoriasis or eczema.

9. Use of an antibody that neutralizes interleukin-8, for the manufacture of a composition as defined in any of claims 1 to 7, for treating an inflammatory skin condition.

10. Use according to claim 9, wherein the condition is psoriasis.

Patentansprüche

1. Pharmazeutische Zusammensetzung für die topische Verabreichung, um einen Hautentzündungszustand zu behandeln, welche einen Antikörper umfasst, der Interleukin-8 neutralisiert.

2. Zusammensetzung nach Anspruch 1, in welcher der Antikörper ein monoklonaler Antikörper ist.

3. Zusammensetzung nach Anspruch 1, in welcher der Antikörper ein polyklonaler Antikörper ist.

4. Zusammensetzung nach Anspruch 1, in welcher der Antikörper ein Antikörper-Fragment ist.

5. Zusammensetzung nach Anspruch 1, umfassend mindestens einen der Antikörper 18-60 (ATCC-Zugangsnr. CRL-12528), 18-S2 (ATCC-Zugangsnr. CRL-12527) und 3C6 (ATCC-Zugangsnr. CRL-12529).

6. Zusammensetzung nach irgendeinem vorangehenden Anspruch, die einen pharmazeutisch annehmbaren Träger umfasst, der aus einer neutralen sterilen Creme, Salbe, einem neutralen sterilen Gel, Gallert, Aerosol, Pflaster und Pulver ausgewählt ist.

7. Zusammensetzung nach irgendeinem vorangehenden Anspruch, in welcher der annehmbare Träger eine Creme ist.

8. Zusammensetzung nach irgendeinem vorangehenden Anspruch, in welcher der Zustand Psoriasis oder Ekzem ist.

9. Verwendung eines Antikörpers, der Interleukin-8 neutralisiert, für die Herstellung einer Zusammensetzung, die in irgendeinem der Ansprüche 1 bis 7 definiert ist, zur Behandlung eines Hautentzündungszustandes.

10. Verwendung nach Anspruch 9, bei der der Zustand Psoriasis ist.

Revendications

1. Composition pharmaceutique pour l'administration topique, destinée au traitement d'une affection inflammatoire cutanée, comprenant un anticorps qui neutralise l'interleukine-8.

2. Composition suivant la revendication 1, dans laquelle l'anticorps est un anticorps monoclonal.

3. Composition suivant la revendication 1, dans laquelle l'anticorps est un anticorps polyclonal.

4. Composition suivant la revendication 1, dans laquelle l'anticorps est un fragment d'anticorps.

5. Composition suivant la revendication 1, comprenant au moins un des anticorps 18 - 60 (n° de dépôt ATCC CRL-12528), 18 - S2 (n° de dépôt ATCC CRL-12527) et 3C6 (n° de dépôt ATCC CRL-12529).

6. Composition suivant l'une quelconque des revendications précédentes, qui comprend un support pharmaceutiquement acceptable choisi entre une crème, un gel, une gelée, une pommade, un aérosol, un timbre et une poudre stériles, neutres.

7. Composition suivant l'une quelconque des revendications précédentes, dans laquelle le support acceptable est une crème.

8. Composition suivant l'une quelconque des revendications précédentes, dans laquelle l'affection est le psoriasis ou l'eczéma.

9. Utilisation d'un anticorps qui neutralise l'interleukine-8, pour la production d'une composition répondant à la définition suivant l'une quelconque des revendications 1 à 7, à des fins de traitement d'une affection inflammatoire cutanée.

10. Utilisation suivant la revendication 9, dans laquelle l'affection est le psoriasis.

Immunoglobulin subtypes of Anti-IL-8 Monoclonal Antibodies	
Clone No.	Subclass
I8-S1	IgG2b, kappa
I8-S2	IgG2b, kappa
I8-60	IgG1, kappa
I8-61	IgG1, kappa
I8-62	IgG1, kappa
I8-63	IgG1, kappa
I8-64	IgG1, kappa
I8-65	IgG, kappa
3C6	IgG1, kappa

FIGURE 1

2nd Reaction Clones	1st Reaction Clones		
	I8-S2 (AI%)	I8-60 (AI%)	3C6(AI%)
I8-S2	-	100.3	56.7
I8-60	90.6	-	108.1
3C6	84.3	74.4	-

FIGURE 2

Dimethyl silicon oil	20 g
Liquid paraffin	10 g
Stearic acid	10 g
Cetyl alcohol	1 g
Stearic alcohol	3 g
Glycerine	20 g
Ethylparaben, Ethyl-parahydroxy-benzoate nipagin A	0.1 g
Higher fatty alcohol-epoxy ethane condensed polymer	0.36 g
Softening agent SG (stearo-epoxy ethane condensed polymer)	0.84 g

FIGURE 3

Type	No. of cases	Results
Plaque psoriasis	23	12 effective, 11 non-effective
Erythrodermic psoriasis	4	4 obvious effect
Arthritic psoriasis	2	2 obvious effect
Total	29	12 effective, 6 obvious effect, 11 non-effective

FIGURE 4

Type	No. of cases	Results
Plaque psoriasis	4	2 obvious effect, 2 non-effective
Erythrodermic psoriasis	2	2 obvious effect
Arthritic psoriasis	2	2 obvious effect
Total	8	6 obvious effect, 2 non-effective

FIGURE 5

Type	No. of cases	Results
Eczema	8	5 obvious effect, 3 non-effective

FIGURE 6

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